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(54) Title: SCREENING FOR CYTOKINE MODULATORS

(57) Abstract

This invention provides a method for screening for agents useful for treatment of diseases and pathological conditions affected by cytokines. These agents interact directly or indirectly with an intracellular receptor, which in turn modulates the binding of a rel-like protein, a rel-like protein complex, or other transcriptional proteins to a rel site on the promoter of a cytokine gene. The intracellular receptor can be the estrogen receptor, retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, or vitamin D receptor. The select agents can be used to treat osteoporosis, rheumatoid arthritis, inflammation, psoriasis, Kaposi's sarcoma, septic shock and multiple myeloma.

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SCREENING FOR CYTOKINE MODULATORS

FIELD OF THE INVENTION

This invention relates to a method for screening for agents useful for treatment of diseases and pathological conditions affected by cytokines and novel agents identified using such screening method.

BACKGROUND OF THE INVENTION

Cytokines are a group of molecules capable of signalling cellular development. Aberrant expression of cytokines is known to be associated with pathological conditions including autoimmune diseases, septic shock, rheumatoid arthritis, psoriasis, inflammation, postmenopausal osteoporosis, and some cancers. Common treatment for these pathological conditions are retinoids, immunosuppressants, glucocorticoids and other steroid drugs. Estrogens are specifically employed in the prevention of postmenopausal osteoporosis.

Steroids and related hormone drugs exert their therapeutic effects by binding to a superfamily of intracellular receptors (IRs), which are regulators of gene transcription. IRs can function as activators as well as repressors of specific cytokine genes. The activity of IRs is controlled by hormones or other ligands that bind to the IRs.

The classical mechanism of transcriptional regulation by IRs involves binding of the IRs to specific response elements in the promoters of the regulated genes, for example, the binding of the estrogen receptor to its response site in the vitellogenin gene (Klein-Hitpass et al., Cell 46:1053-1061, 1986). More recently a different mechanism of IRs function has been described in glucocorticoid receptor mediated AP-1 transcription regulation that does not

require direct DNA-binding of the IRs (Yang-Yen et al., Cell 62:1205-1215, 1990).

Although steroid drugs have been shown to repress the level of certain cytokines, a lack of tissue specificity and side effects of the steroids may limit their use as therapeutic agents. These side effects may be reduced or completely avoided with more specifically acting compounds.

Pfahl and Karin (PCT publication, WO 92/07072, 1992) describes a method of screening a sample for ligands which bind to a nuclear receptor to form a complex which binds or interferes with a non rel-like protein AP-1 or an AP-1 component.

SUMMARY OF THE INVENTION

The present invention relates to a method for identifying new therapeutic agents and for using these agents to treat diseases and conditions affected by cytokines, such as, but not limited to, osteoporosis, rheumatoid arthritis, inflammation, psoriasis, septic shock, Kaposi's sarcoma and multiple myeloma. This method makes it possible to screen large collections of natural, semisynthetic, or synthetic compounds for therapeutic agents that affect the transcription of a cytokine through an intracellular receptor mediated pathway.

By "cytokine" is generally meant a secreted protein which acts as a chemical mediator of cellular regulation.. More specifically, it is meant a diverse groups of soluble polypeptides such as growth factors and hormones that control the growth, differentiation and function of cells, including, but not limited to, GM-CSF, G-CSF, IL-2, IL-6, IL-8, and IL-11.

The present invention relates to the determination that inhibition of interleukin 6 (IL-6) expression by estrogen-estrogen receptor complex is mediated through

the control of the transcriptional activity of NFκB or closely related proteins on the IL-6 promoter. This mechanism does not involve direct binding of ER to IL-6 promoter but controls the DNA-binding properties of the activated NFκB and possible other members of the rel-family of proteins to their specific response elements (i.e., rel site) on the IL-6 promoter.

Because NFκB is involved in the regulation of genes encoding various cytokines and their receptors, viral proteins, and proteins involved in the acute-phase response, the regulation of NFκB activity by estrogen and possible other hormones is of general importance (see generally Baeuerle, Biochemica et Biophysica Acta, 1072:63-80, 1993, incorporated by reference herein). For example, retinoic acid treatment, which strongly inhibits IL-6 expression in +/+LDA11 cells and other tissues (Gross, V., P. M. Villiger, B. Zhang, and M. Lotz, 1993, "Retinoic acid inhibits interleukin-1-induced cytokine synthesis in human monocytes," J. Leukoc. Biol. 54:125-132), has the same effect as estrogen on the NFκB related complexes with the IL-6 promoter. This suggests a general pathway of transcriptional regulation involving cross-talk between members of the intracellular receptor family and the NFκB transcription factors.

The above determination allows for the screening of drugs that specifically influence genes controlled by the rel-transcription factors, i.e. genes involved in inflammation, sepsis, skin and kidney disorders, osteoporosis, certain cancers, and hematopoietic dysfunctions without the side effects of known steroid drugs. The diseases listed are usually correlated with aberrant expression of cytokines such as IL-1, TNFα, IL-6, IL-8 that are under the control of NFκB or other rel proteins.

Thus, the present invention features a method for identifying agents which, by activating an intracellular receptor, cause a significant reduction in the binding of a rel-like protein or other transcriptional protein to the rel site on the promoter of a cytokine gene or a portion of the promoter, thereby reducing the transcription of the cytokine.

By "intracellular receptor" is meant an intracellular transcription factor whose activity is regulated by binding of small molecules, including, but not limited to, estrogen receptor, retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, and vitamin D receptor.

By "rel-like protein" is meant a protein or a protein complex of the rel family that share a homology in the rel domain and is involved in gene regulation (see Liou and Baltimore, Current Opinion in Cell Biology, 5:477-487, 1993, incorporated by reference herein), including, but not limited to, NFκB, Lyt-10, c-rel, and relB.

By "transcriptional protein" is meant a cytoplasmic or nuclear protein that, when activated, bind a promoter either directly, or indirectly through a complex of proteins to modulate the transcription activity of the promoter.

By "rel site" is meant a DNA sequence that serves as a binding site for rel-like proteins or complexes comprising one or more rel-like proteins, including, but not limited to, κB motifs identified in Baeuerle, Biochemica et Biophysica Acta, 1072:63-80, 1993, incorporated by reference herein, such as the NFκB binding site on IL-6 promoter.

By "promoter" is meant a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a

downstream (3' direction) coding sequence. A promoter of a DNA construct, including an oligonucleotide sequence according to the present invention may be linked to a heterologous gene when the presence of the promoter influences transcription from the heterologous gene, including genes for reporter sequences such as luciferase, chloramphenicol acetyl transferase, β-galactosidase and secreted placental alkaline phosphatase.

In a preferred embodiment, the assay is conducted in a whole cell system that has an intracellular receptor which is the target of the screened agent, a promoter or a portion of a promoter with a rel site and a rel-like protein or other transcription protein that binds to the rel site; wherein the intracellular receptor modulates the binding of the rel-like protein or the transcription protein to the rel site. The intracellular receptor, the promoter or a portion of the promoter, or the protein that binds to the rel site may either be endogenous to the cell or transfected into the cell.

In another preferred embodiment, the assay is conducted in an extract of cell having an intracellular receptor, a promoter or a portion of a promoter, with a rel site and a rel-like protein or other protein that binds to the rel site; wherein the intracellular receptor modulates the binding of the rel-like protein or the transcription protein to the rel site.

The binding of the rel-like protein or other transcription protein to the rel site may be measured by techniques known to those skilled in the art, including, but not limited to, mobility shift assay, co-transfection assay, and expression of a reporter gene linked to the promoter.

In a further preferred embodiment, the promoter is activated by an effector, including, but not limited to, tumor necrosis factor, interleukin-1, viruses,

endotoxins, phorbol esters, epidermal growth factor, leukemia inhibitor factor and cAMP agonists.

By "effector" is meant an agent that stimulates the expression of a cytokine to a measurable level. An effector may be endogenously produced in a cell or exogenously added to a cell

In another further preferred embodiment, the claimed assay is conducted in a system including an estrogen receptor, an interleukin 6 promoter or a portion of an IL-6 promoter and NFκB; wherein ER modulates the binding of NFκB or related proteins to the NFκB site on the IL-6 promoter.

The agents discovered by the above assay may either interact directly with an intracellular receptor, or modulate the interaction of a ligand with the intracellular receptor. Thus, in an even further preferred embodiment, a ligand for the intracellular receptor is included in the assay.

While steroids and steroid analogs may exemplify agents identified by the present invention, Applicant is particularly interested in the identification of agents of low molecular weight (less than 10,000 daltons, preferably less than 5,000, and most preferably less than 1,000) which can be readily formulated as useful therapeutic agents.

Such agents can then be screened to ensure that they are specific to tissues with cytokine inflicted pathological conditions with little or no effect on healthy tissues such that the agents can be used in a therapeutic or prophylactic manner. If such agents have some effect on healthy tissues they may still be useful in therapeutic treatment, particularly in those diseases which are life threatening, such as Kaposi's sarcoma or multiple myeloma.

Once isolated, a candidate agent can be put in pharmaceutically acceptable formulations, and used for

specific treatment of diseases and pathological conditions with little or no effect on healthy tissues.

Other features and advantages of the invention will be apparent from the following detailed description of the invention, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows IL-1 and TNFα induced complex formation on the proximal IL-6 promoter.

Figure 2 shows that several distinct NFκB-related complexes induced by IL-1 and TNFα are modulated by estrogen.

Figure 3 shows the effects of estrogen agonist and antagonist, and inhibitors of protein synthesis and protein kinase C on the formation of NFκB-related complexes.

Figure 4 shows the binding characteristics of proteins in NFκB-related complexes with NFκB oligonucleotides.

Figure 5 shows NFκB related proteins in complexes A, B, and C.

DETAILED DESCRIPTION OF THE INVENTION

A number of cytokines, including IL-6, Il-8 and IL-11, have related biological effects, i.e., effects on cellular defense in response to infection by stimulating the immune and the acute-phase response and on bone metabolism by increasing bone resorption. Aberrant expression of any of these cytokines results in similar pathological conditions, e.g., all cytokines listed are involved in septic shock. In another example, excessive production IL-8, like IL-6, may be involved in the pathogenesis of several types of inflammatory reactions, particularly neutrophil-dependent tissue damages. These cytokines have similar promoter structures, e.g., their promoters contain binding sites for NFκB or other rel

proteins. It is therefore likely that not only IL-6 but also the other cytokines mentioned above can be targeted by drugs that modulate the binding of NFκB or other rel proteins to their promoter sites through the intracellular receptors.

Interleukin 6 and Diseases

Interleukin-6 (IL-6) is a pleiotropic cytokine that is secreted by many different cells, including monocytes, macrophages, certain B-lymphocytes and T-lymphocytes, glial cells, fibroblasts, osteoblasts, and stromal cells (reviewed in references Hirano, T., (1992) "The biology of interleukin-6," Chem. Immunol. 51:153-180.; Kishimoto, T. (1989) "The biology of interleukin-6," Blood 74:1-10.; Kishimoto, T., M. Hibi, M. Murakami, M. Narazaki, M. Saito, and T. Taga (1992) "The molecular biology of interleukin 6 and its receptor," Ciba Found. Symp. 167:5-16; discussion 16-23; and Wolvekamp, M. C., and R. L. Marquet (1990) "Interleukin-6: historical background, genetics and biological significance," Immunol. Lett. 24:1-9). Due to its induction in response to tissue injury, inflammation and infection, IL-6 function is mainly associated with the host's immune and acute phase responses.

IL-6 is an important mediator of intercellular communication not only under pathological conditions but also under normal physiological conditions. It is involved in neural differentiation (Satoh, T., S. Nakamura, T. Taga, T. Matsuda, T. Hirano, T. Kishimoto, and Y. Kaziro (1988) "Induction of neuronal differentiation in PC12 cells by B-cell stimulatory factor 2/interleukin 6," Mol. Cell Biol. 8:3546-3549), and proliferation and differentiation during hematopoiesis (Ikebuchi, K., G.G. Wong, S.C. Clark, J.N. Ihle, Y. Hirai, and M. Ogawa (1987) "Interleukin 6 enhancement of interleukin 3-dependent proliferation of

multipotential hemopoietic progenitors," Proc. Natl. Acad. Sci. U.S.A. 84:9035-9039). However, elevated IL-6 expression is usually associated with disease (Yu, X.P., T. Bellido, N. Rice, and S.C. Manolagas (1993).

IL-6 expression is tightly controlled by other factors. Depending on the particular cell type, it can be activated by various stimuli, including tumor necrosis factor (TNFα) and interleukin-1 (IL-1), viruses, endotoxin (lipopolysaccharides), phorbol esters, epidermal growth factor (EGF), leukemia inhibitor factor (LIF), and cAMP agonists.

These effectors exhibit their activity through transcriptional effects on the IL-6 promoter as shown by transfection studies (Gruss, H.J., M.A. Brach, and F. Herrmann (1992) "Involvement of nuclear factor-kappa B in induction of the interleukin-6 gene by leukemia inhibitory factor," Blood 80:2563-2570; Ray, A., S.B. Tatter, L.T. May, and P.B. Sehgal (1988) "Activation of the human "beta 2-interferon/hepatocyte-stimulating factor/interleukin 6" promoter by cytokines, viruses, and second messenger agonists," Proc. Natl. Acad. Sci. U.S.A. 85:6701-6705). By sequence comparison several potential transcriptional control elements have been identified in the IL-6 promoter, including a cAMP response element, an AP-1 binding site, and binding elements for the transcription factors NF-IL6 (C/EBPB, LAP, AGP/EBP) and NFκB (Isshiki, H., S. Akira, O. Tanabe, T. Nakajima, T. Shimamoto, T. Hirano, and T. Kishimoto (1990) "Constitutive and interleukin-1 (IL-1)-inducible factors interact with the IL-1-responsive element in the IL-6 gene," Mol. Cell Biol. 10:2757-2764).

Direct binding of NF-IL6 and NFκB to the IL-6 promoter has been established (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A

nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Libermann, T.A. and D. Baltimore, (1990) "Activation of interleukin-6 gene expression through the NF-kappa B transcription factor," Mol. Cell Biol. 10:2327-2334). NF-IL6 belongs to the C/EBP family of leucine zipper proteins. It is induced by IL-1, IL-6 and lipopolysaccharide (LPS), and has been shown to interact with its binding site on the IL-6 promoter and to activate IL-6 expression (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto, (1990) "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Chang, C.J., T.T. Chen, H.Y. Lei, D.S. Chen, and S.C. Lee (1990), "Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family," Mol. Cell Biol. 10:6642-6653; Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler (1990) "LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein," Genes Dev. 4:1541-1551; Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler (1990) "LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein," Genes Dev. 4:1541-1551; Poli, V., F.P. Mancini, and R. Cortese (1990) "IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP," Cell 63:643-653). NFκB is a transcription factor that was originally identified as a heterodimeric complex consisting of a 50 kD protein (p50) and a 65 kd protein (p65) that binds an element in the immunoglobulin kappa light chain enhancer. Both proteins reveal a high

homology to the Drosophila morphogen *dorsal* and to the c-rel proto-oncogeny product. The p65 subunit is also functionally related to c-rel (reviewed in references Baeuerle, P. A. (1991) "The inducible transcription activator NF-kappa B: regulation by distinct protein subunits" Biochim. Biophys. Acta 1072:63-80; Blank, V., P. Kourilsky, and A. Israel (1992) "NF-kappa B and related proteins: Rel/dorsal homologies meet ankyrin-like repeats," Trends. Biochem. Sci. 17:135-140; and Liou, H.C. and D. Baltimore (1993) "Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system," Curr. Opin. Cell Biol. 5:477-487). Recently, additional proteins (p49/p52 and relB/p68) have been identified that are functionally related to p50 and p65 (Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle (1993) "Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B," Nature 365:182-185; Perkins, N.D., R.M. Schmid, C.S. Duckett, K. Leung, N.R. Rice, and G.J. Nabel (1992) "Distinct combinations of NF-kappa B subunits determine the specificity of transcriptional activation," Proc. Natl. Acad. Sci. U.S.A. 89:1529-1533; Ryseck, R.P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo (1992) "RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B," Mol. Cell Biol. 12:674-684; Ryseck, R.P., P. Bull, M. Takamiya, V. Bours, U. Siebenlist, P. Dobrzanski, and R. Bravo (1992) "RelB, a new Rel family transcription activator that can interact with p50-NF-kappa B," Mol. Cell Biol. 12:674-684; Schmid, R.M., N.D. Perkins, C.S. Duckett, P.C. Andrews, and G.J. Nabel (1991) "Cloning of an NF-kappa B subunit which stimulates HIV transcription in synergy with p65," Nature 352:733-736). NFκB is located in the cytosol complexes with an inhibitory protein of the IκB family. Upon induction, NFκB

dissociates from IκB and translocates into the nucleus where it binds and activates specific promoters (Baeuerle, P.A. and D. Baltimore (1988) "I kappa B: a specific inhibitor of the NF-kappa B transcription factor," Science 242:540-546; Ghosh, S. and D. Baltimore (1990) "Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B," Nature 344:678-682). Binding of NFκB-like factors to the consensus site of the IL-6 promoter is induced by IL-1, TNFα, LIF, LPS and phorbol esters, varying with the particular cell type (Gruss, H.J., M.A. Brach, and F. Herrmann (1992) "Involvement of nuclear factor-kappa B in induction of the interleukin-6 gene by leukemia inhibitory factor," Blood 80:2563-2570; Libermann, T.A. and D. Baltimore (1990) "Activation of interleukin-6 gene expression through the NF-kappa B transcription factor," Mol. Cell Biol. 10:2327-2334; Shimizu, H., K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1990) "Involvement of a NF-kappa B-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines," Mol. Cell Biol. 10:561-568; Zhang, Y.H., J.X. Lin, and J. Vilcek (1990) "Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence," Mol. Cell Biol. 10:3818-3823).

Unregulated expression of IL-6 is linked to a number of diseases (Bauer, J. and F. Herrmann (1991) "Interleukin-6 in clinical medicine," Ann. Hematol. 62:203-210; Hirano, T. (1992) "Interleukin-6 and its relation to inflammation and disease," Clin. Immunol. Immunopathol. 62:S60-S65) such as postmenopausal osteoporosis after loss of ovarian function (Roodman, G.D. (1992) "Interleukin-6: an osteotropic factor?" J. Bone Miner. Res. 7:475-478). Ex vivo cultures of bone marrow from ovariectomized mice show an increase of

osteoclastogenesis compared with cultures from sham-operated animals. This increase in osteoclast development can be prevented by injection of an anti-IL-6 antibody or by administration of estrogen (Jilka, R.L., G. Hangoc, G. Girasole, G. Passeri, D.C. Williams, J.S. Abrams, B. Boyce, H. Broxmeyer, and S.C. Manolagas (1992) "Increased osteoclast development after estrogen loss: mediation by interleukin-6," Science 257:88-91). In mice that carry a null mutation for IL-6, ovariectomy does not affect bone volume or osteoclast number as seen with normal mice (Balena, R., F. Costantini, M. Yamamoto, A. Markatos, R. Cortese, G.A. Rodan, and V. Poli (1993) "Mice with IL-6 gene knock-out do not lose cancellous bone after ovariectomy," J. Bone Miner. Res. 8:S130 [Abstract]).

Regulation of Interleukin 6 by Estrogen

Estrogen has been found to inhibit IL-6 expression in bone-derived stromal cell lines and osteoblastic cells from rats and mice as well as in nontransformed human bone cells (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891). This effect of estrogen on IL-6 expression is not restricted to bone tissue but has also been shown for uterine cells (Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046; Tabibzadeh, S.S., U. Santhanam, P.B. Sehgal, and L.T. May (1989) "Cytokine-induced production of IFN-beta 2/IL-6 by freshly explanted human endometrial stromal cells. Modulation by estradiol-17 beta," J. Immunol. 142:3134-

3139). There are only a few other genes known to be negatively regulated by estrogen agonists (Adler, S., M.L. Waterman, X. He, and M.G. Rosenfeld (1988) "Steroid receptor-mediated inhibition of rat prolactin gene expression does not require the receptor DNA-binding domain," Cell 52:685-695; Ree, A.H., B.F. Landmark, W. Eskild, F.O. Levy, H. Lahooti, T. Jahnsen, A. Aakvaag, and V. Hansson (1989) "Autologous down-regulation of messenger ribonucleic acid and protein levels for estrogen receptors in MCF-7 cells: an inverse correlation to progesterone receptor levels," Endocrinology 124:2577-2583).

To investigate the mechanism of the estrogen effect, Applicant performed a series of DNA-binding experiments using the human IL-6 promoter. Co-transfection studies showed that the proximal 225 bps of the IL-6 promoter mediate both the induction of the reporter gene by IL-1 and TNFα as well as the repression by estradiol. The repression by estradiol also required the expression of the estrogen receptor (ER).

Using gel retardation assays, no specific binding of the ER to the proximal 225 bp could be detected. However, nuclear extracts from +/+LDA11 bone marrow stromal cells that revealed IL-6 regulation by IL-1, TNFα, and estradiol showed an induced complex with a -225 to -52 promoter fragment when the cell were treated with IL-1 and TNFα. Induction of the complex was fast (10 minutes) but transient. Pretreatment of the cells with estradiol increased the intensity as well as the mobility of the complex.

To identify the proteins involved in the formation of the complex, antibody supershift experiments were carried out using antibodies against factors with potential binding sites in this promoter fragment including c-jun, NF-IL6, c-rel, and NFκB p50 and p65

proteins. Only anti-p50 and anti-p65 had an effect and abolished the formation of the induced complex.

An oligonucleotide covering the potential NFκB site of the IL-6 promoter competed against the induced binding to this fragment, while an oligonucleotide covering the NF-IL6 site was ineffective. When the NFκB oligonucleotide was used as probe, three IL-1/TNFα-induced complexes were observed.

Pretreatment with estradiol decreased the intensity of the slowest complex and strongly increased the intensity of the fastest migrating complex. The three bands were differentially supershifted (i.e., further decrease in the mobility of the complex due to binding of the antibody) by anti-p50 and anti-p65 antibodies, while none of several other antibodies tested, including anti-ER antibody, had any effect. Methylation interference assays showed identical DNA contact sites for all three complexes.

Ray, et al., J. Biol. Chem., 269(17):12940-946 (1994), not admitted to be prior art, describe that activation of the IL-6 promoter, elicited by a combination of NF-IL6 and the p65 subunit of NFκB, can be inhibited by the wt ER but not by an ER containing a mutation in its DNA binding domain. Furthermore, the repression of the IL-6 promoter by a combination ER and 17β-estradiol did not appear to be mediated via high affinity binding of the receptor to the promoter.

These data suggest that negative regulation by estrogen is mediated through the IL-6 promoter and is estrogen receptor dependent. Inhibition of IL-6 expression by estrogen is mediated through control of the transcriptional activity of NFκB or closely related proteins on the IL-6 promoter.

Mukaida, et al., J. Biol. Chem., 269(18):13289-295 (1994), not admitted to be prior art, describe that a glucocorticoid, dexamethasone, inhibited IL-8 production

at the transcriptional level. Mutation of either the AP-1 or NF-IL6 binding site on the IL-8 promoter did not abolish IL-8 gene repression by dexamethasone, suggesting that these sites were not targets for dexamethasone. Yet dexamethasone diminished the IL-1 induced formation of NFκB complexes.

The invention will now be described in greater detail by reference to the following non-limiting examples regarding the regulation of interleukin 6 transcription by estrogen receptor.

Examples

A candidate agent will be screened by either A) direct evaluation of protein binding to rel-sites, or B) indirect evaluation of binding to rel-sites.

A) Direct evaluation of protein binding to rel-sites

Cells selected for expression of the necessary components will be treated with the agent or vehicle control and an inducer (e.g., phorbol ester, cytokines, lipopolysaccharides). Cellular extracts prepared from those cells (e.g., whole cell, cytosolic, or nuclear extracts) will be analyzed for their DNA-binding using cytokine promoter fragments or various rel-sites as probes. Binding will be analyzed qualitatively (i.e., comparing pattern) and quantitatively comparing extracts from cells treated with vehicle or the agent.

B) Indirect evaluation of binding to rel-sites by

1) Measuring endogenous cytokine expression.

Cells selected for expression of the necessary components and their production of cytokine will be treated with the agent or vehicle control and an inducer (phorbol ester, cytokines, lipopolysaccharides). Activity of the agent will be quantitatively assessed by

measuring of cytokine using standard assays known to those skilled in the art.

2) Measuring the expression of a reporter introduced into the cell.

By means of transfection a reporter construct will be introduced into the cells that expresses an easily measurable protein under the control of a cytokine promoter or fragments thereof or isolated rel-sites. The other necessary components are either expressed endogenously by the cells or provided by cotransfection of expression vectors for the particular component. Cells will be treated with the agent or vehicle control and an effector (phorbol ester, cytokines, lipopolysaccharides). The activity of the agent will be analyzed quantitatively by measuring the expression of the reporter protein.

Agents will also be tested for their binding to IRs by traditional binding assays as well as for their activity to effect the classical mechanism of gene regulation by IRs. An agent that binds to IRs and regulates binding of rel proteins to cytokine promoters but does not activate the classical mechanism of IR action is a potential drug candidate for the specific treatment of diseases associated with aberrant expression of cytokines.

Experimental procedures employed in the examples described herein are set forth below:

Transient transfections and mammalian expression constructs

Construction of the pERE-tk-Luc reporter plasmid and the vector expressing ER_{gly} (pRShER) has been described (Tzukerman, M., A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, and D.P. McDonnell (1994) "Human estrogen receptor transactivational capacity is determined by both cellular and promoter

context and mediated by two functionally distinct intramolecular regions," Mol. Endocrinol. 8:21-30, incorporated by reference herein). The pIL6[-225]Luc reporter construct was derived from the parental pIL6[-1200]Luc by excision of a NheI-BamHI fragment and religation of the vector fragment after blunt ending with Klenow DNA-polymerase. The parental pIL6[-1200]Luc was constructed by cloning the 1.2 kb IL-6 promoter insert excised with BamHI and KpnI from pCAT-M54-IL6(-) into the corresponding sites of the luciferase vector Lucpl.

C3H10T1/2 cells were seeded in phenol-red-free DMEM supplemented with 10% FBS at 80,000 cells per well (12-well plates). The cells were transfected by calcium phosphate precipitation (Peterson, J.L. and O.W. McBride (1980) "Cotransfer of linked eukaryotic genes and efficient transfer of hypoxanthine phosphoribosyltransferase by DNA-mediated gene transfer," Proc. Natl. Acad. Sci. U.S.A. 77:1583-1587) with 0.5 mg pIL6[-225]Luc alone or together with 0.05 mg pRShER or 0.1 mg HE0 using pGEM as carrier to adjust to 2 mg total DNA in the transfection mix. After 4 h at 37 °C the cells were treated with 7% DMSO for 30 min followed by a medium change and addition of hormones. The following day the cells were induced with TNFα and IL-1b (1 nM each) for 24 h. After a brief wash with PBS the cells were lysed in 200 ml lysis buffer (25 mM Tris [pH 7.8], 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N',N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100). To 20 ml of each extract 100 ml of reagent (20 mM Tricine [pH 7.8], 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 279 mM coenzyme A, 470 mM luciferin, 530 mM ATP) was added and luciferase activity was measured immediately with a Dynatech luminometer in cycle mode.

Antibodies, IL-6 ELISA, and ER assay

Peptides used to raise the following antibodies in rabbits correspond to amino acid residues 91-105 of murine c-jun (Ryder and Nathans (1988) "Induction of protooncogene c-jun by serum growth factors," Proc. Natl. Acad. Sci. USA 85:8464-8467), 278-296 of murine NF-IL6 (Chang, C.J., T.T. Chen, H.Y. Lei, D.S. Chen, and S.C. Lee (1990) "Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family," Mol. Cell Biol. 10:6642-6653), 152-176 of murine c-rel (Bull, P., K.L. Morley, M.F. Hoekstra, T. Hunter, and I.M. Verma (1990) "The mouse c-rel protein has an N-terminal regulatory domain and a C-terminal transcriptional transactivation domain," Mol. Cell Biol. 10:5473-5485; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. U.S.A. 88:3715-3719), 347-361 of murine p50 (Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan, and D. Baltimore (1990) "Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal," Cell 62:1019-1029), and 3-19 of human p65 (88% homology with murine p65) (Nolan, G.P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore (1991) "DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide," Cell 64:961-969; Ruben, S.M., P.J. Dillon, R. Schreck, T. Henkel, C.H. Chen, M. Maher, P.A. Baeuerle, and C.A. Rosen (1991) "Isolation of a rel-related human cDNA that potentially encodes the 65-kD subunit of NF-kappa B [letter]," Science 254:11). All the references mentioned above are incorporated by reference herein. All antibodies listed above were obtained affinity purified at a concentration of 1 mg/ml from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TBP was a protein-A purified serum preparation from

a rabbit immunized with the full length human recombinant protein and reported to react with TBP from mouse, rat, and human origin (Santa Cruz Biotechnology, Inc.). Anti-ER is a mouse monoclonal antibody (IgG2a) raised against a peptide corresponding to amino acid residues 8-22 of the murine ER. IL-6 concentration in tissue culture supernatants was determined by use of an IL-6 ELISA kit (Endogen, Inc., Boston, MA) using murine IL-6 as standard.

ER in +/+LDA11 cells was measured in whole cell extracts. After washing and counting, cells were homogenized in buffer containing 50 mM Tris [pH 7.5], 30% glycerol, 500 mM KCl, 1 mM EDTA, 1 mM PMSF, and 5 mM DTT. After 30 min on ice the homogenate was centrifuged (100,000 g, 4 °C, 1 h). The supernatant was taken as whole cell extract, adjusted to 0.5% CHAPS, and incubated with 5 nM [^{3}H]estradiol in the absence or presence of a 200-fold excess of DES overnight at 4 °C. After incubation with anti-ER antibody, the complexes formed were precipitated with protein-A sepharose (Pharmacia), washed three times with 10 mM Tris [pH 7.5]/0.5% CHAPS, and measured by liquid scintillation counting.

Electrophoretic mobility shift assay (EMSA) and methylation interference assay

DNA binding studies were carried out with nuclear extracts from +/+LDA11 cells, extracts from yeast expressing recombinant human ER_{gly}, and purified p50 and p49 proteins. +/+LDA11 cells were maintained under conditions as described (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens,"

J. Clin. Invest. 89:883-891). To prepare nuclear extracts the cells were seeded in phenol-red-free McCoy's medium supplemented with 10% FBS and pretreated with hormone for 24 h if not indicated otherwise. After adjusting the medium to 2% FBS, the cells were induced with TNFα and IL-1b (1 nM each) for varying periods. In cases where cycloheximide (10 mg/ml) or the kinase inhibitor H7 (50 mM) were included, those compounds were added 5 min before induction. Incubation was stopped by two washes with ice cold PBS and cells were lysed in situ in cold buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% Nonidet P-40). Lysates were transferred into microfuge tubes, nuclei pelleted (8000 rpm, 1 min) and resuspended in buffer C (20 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF). After 40 min rocking at 4 °C, samples were centrifuged (15,000 rpm, 10 min) and supernatants taken as nuclear extracts. Bradford protein assays (Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," Anal. Biochem. 72:248-254) showed only minimal variations in protein concentrations which did not correlate with hormone or cytokine treatment. Extracts of yeast recombinantly expressing ER_{gly} were prepared from the BJ2168 strain transformed with YEpE10 as described (Tzukerman, M., A. Esty, D. Santiso-Mere, P. Danielian, M.G. Parker, R.B. Stein, J.W. Pike, and D.P. McDonnell (1994) "Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions," Mol. Endocrinol. 8:21-30). Purified, *Escherichia coli* expressed human p50 and p49 proteins were purchased from Promega (Madison, WI).

For EMSA, 2ml of the extracts were preincubated with 2 mg poly[dI-dC] in binding buffer adjusted to 20 mM HEPES [pH 7.9], 40 mM NaCl, 20 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.1 mg/ml BSA, and 1 mM DTT. When the -225 to -52 IL-6 promoter fragment was used as probe 0.5 mg of Bluescript plasmid (Stratagene, La Jolla, CA) was also included. After 20 min on ice, the probe was added and the incubation continued for 20 min at room temperature. When antibodies were included, 1 mg was added 20 min after the probe and the incubation continued for 40 min at 4 °C. The complexes formed were analyzed on non-denaturing polyacrylamide gels (4% acrylamide/0.05% BIS; 2x200 mm) at 4 °C and 15 V/cm in 0.25xTBE. Probes were either double stranded oligonucleotides corresponding to the regions -82 to -47 (ATCAAATGTGGGATTTTCCCATGAGTCTCAATATTA) and -172 to -131 (CTAAAGGACGTCACATTGCACAATCTTAATAAGGTTTCCAAT) of the human IL-6 promoter and to the ERE of the vitellogenin promoter (Tzukerman, M., X.K. Zhang, T. Hermann, K.N. Wills, G. Graupner, and M. Pfahl (1990) "The human estrogen receptor has transcriptional activator and repressor functions in the absence of ligand," New Biol. 2:613-620) or the -225 to -52 NheI-SspI IL-6 promoter fragment. All probes were either labeled with [γ^{32}P]ATP using T4-polynucleotide kinase or with [α^{32}P]dATP using Klenow polymerase and subsequently purified by polyacrylamide gel electrophoresis.

For methylation interference assays, the -82 to -47 probe labeled with [γ^{32}P]ATP either on the upper or the lower strand was subjected to limited DMS-methylation (Maxam, A.M. and W. Gilbert (1980) "Sequencing end-labeled DNA with base-specific chemical cleavages," Methods Enzymol. 65:499-560). EMSA was performed as described above, scaled up 10-fold. Gels were blotted onto NA45 anion exchange membranes (Schleicher & Schuell) in 0.5xTBE for 30 min at 30 V (Singh, H., J.H.

LeBowitz, A.S. Baldwin, Jr., and P.A. Sharp (1988) "Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA," Cell 52:415-423). After autoradiography, the DNA corresponding to the various complexes and the unretarded probe was eluted (10 min at 65 °C in 20 mM Tris [pH 8.0], 1 M NaCl, 0.1 mM EDTA) and purified by phenol/chloroform extraction and ethanol precipitation. After strand cleavage in 1 M piperidine (30 min at 90 °C) the fragments were resolved on denaturing polyacrylamide gels (12% acrylamide/0.6% BIS).

Example 1. Screening for ER mediated inhibition of IL-6 promoter activity

It has been shown that IL-6 repression is regulated by estradiol at the mRNA level (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891; Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046). To determine if estrogen or a candidate agent acts directly on IL-6 transcription, we transfected a reporter construct, expressing the firefly luciferase under the control of the human IL-6 promoter region from -225 to +14, into the murine fibroblast cell line C3H10T1/2. These cells can be considered as pre-osteoblasts since they differentiate into osteogenic cells in response to bone morphogenic protein-2 (Katagiri, T., A. Yamaguchi, T. Ikeda, S. Yoshiki, J.M. Wozney, V. Rosen, E.A. Wang, H.

Tanaka, S. Omura, and T. Suda (1990) "The non-osteogenic mouse pluripotent cell line, C3H10T1/2, is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2," Biochem. Biophys. Res. Commun. 172:295-299).

Therefore, C3H10T1/2 cells were transfected with a luciferase expression vector under the control of the proximal human IL-6 promoter (pIL6[-225]Luc) alone or together with the expression vector for the wild-type human ER_{gly} (pRShER). After pretreatment with varying concentrations of estradiol for 24 hours, the cultures were induced with 1 nM each of TNFα and IL-1 or left uninduced and 24 h later cells were harvested and extracts analyzed for luciferase activity.

Treatment of transfected cells with IL-1 and TNFα induced a 5-fold increase in luciferase activity over basal levels. Without cotransfection of a plasmid expressing the estrogen receptor, treatment with estradiol had no effect. However, with the expression of estrogen receptor by cotransfection, treatment with estradiol resulted in a strong, dose-dependent repression of luciferase activity.

Repression was observed with the wild-type human ER (ER_{gly}) as well as with an ER variant containing a glycine to valine point mutation in the hormone binding domain (ER_{val}) (Tora, L., A. Mullick, D. Metzger, M. Ponglikitmongkol, I. Park, and P. Chambon (1989) "The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties," EMBO J. 8:1981-1986). While ER_{val} required a higher estradiol concentration, it exhibited a stronger repression. This is consistent with the finding that in induction experiments ER_{gly} responds at lower hormone concentrations but has considerable basal activity (Tzukerman, M., X.K. Zhang, T. Hermann, K.N. Wills, G. Graupner, and M. Pfahl (1990) "The human estrogen receptor has transcriptional

activator and repressor functions in the absence of ligand," New Biol. 2:613-620).

The dependence of the estrogen effect on cotransfected ER suggested that C3H10T1/2 cells do not express functional endogenous ER. This was confirmed by transfecting the cell with a luciferase reporter under the control of the vitellogenin estrogen response element (ERE) (Klein-Hitpass, L., M. Schorpp, U. Wagner, and G.U. Ryffel (1986) "An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells," Cell 46:1053-1061).

Therefore, C3H10T1/2 cells were transfected with a luciferase expression vector under the control of the minimal thymidine kinase promoter and the vitellogenin estrogen response element (pERE-tk-Luc) alone or together with pRShER. 24 h after treatment with 10 nM estradiol or vehicle cells were harvested and extracts analyzed for luciferase activity. Induction of luciferase activity by estradiol was only observed in the presence of cotransfected ER.

In addition, C3H10T1/2 cells were incubated with or without 10 nM estradiol. After 24 h the cultures were induced with TNFα and IL-1 (1 nM each) or left uninduced for additional 24 h. Il-6 in the supernatants was assayed by an ELISA specific for murine IL-6. C3H10T1/2 cells responded to IL-1 and TNFα treatment with strongly increased production of endogenous IL-6, but unlike other osteogenic or stromal cells containing endogenous ER (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891), IL-6 levels were not decreased by estradiol. These data suggest

that the inhibition of IL-6 expression is at the transcriptional level and mediated through the ER.

By cotransfection studies using the preosteoblastic cell line C3H10T1/2, we showed that IL-1/TNFα-induced activation of the proximal IL-6 promoter region could be inhibited by estrogen. This inhibition was estrogen receptor dependent and was observed with both the wild-type human ER (ER_{gly}) and the ER_{val} variant. Similar results have been obtained by others in both HeLa cells cotransfected with ER_{val}, and in MBA13 cells, a preosteoblastic cell line expressing endogenous ER. Together with the described effects of estrogen on IL-6 mRNA (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891; Jacobs, A.L., P.B. Sehgal, J. Julian, and D.D. Carson (1992) "Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro," Endocrinology 131:1037-1046), these results suggest a transcriptional mechanism of estrogen-induced inhibition.

A candidate agent can be screened using the above assay, replacing estradiol with said agent.

Example 2. Screening agents that modulates binding of NFκB related proteins to the proximal IL-6 promoter

A cell line that expresses ER (+/+LDA11)

An exemplary assay system is a cell line that expressed all the necessary components endogenously, including the ER. The bone marrow derived murine stromal cell line +/+LDA11 has been shown to respond to IL-1 and TNFα treatment with strongly increased

secretion of IL-6. Treatment with estradiol inhibits this induction of IL-6 as shown for the protein and its mRNA (Girasole, G., R.L. Jilka, G. Passeri, S. Boswell, G. Boder, D.C. Williams, and S.C. Manolagas (1992) "17 beta-estradiol inhibits interleukin-6 production by bone marrow-derived stromal cells and osteoblasts in vitro: a potential mechanism for the antiosteoporotic effect of estrogens," J. Clin. Invest. 89:883-891).

To verify that ER is actually present in +/+LDA11 cells, hormone binding studies were carried out. Initial experiments showed a low number of specific estradiol binding sites in high salt extracts from these cells. Using a monoclonal antibody directed against the amino terminus of the ER, specifically bound [^{3}H]estradiol was immunoprecipitated confirming that the binding sites represented ER. From those studies we calculated that +/+LDA11 cells contain approximately 1000 ER molecules per cell.

However, when using electrophoretic mobility shift assays (EMSA) in combination with the vitellogenin ERE as a probe, ER-specific DNA binding activity could not be detected in nuclear extracts from +/+LDA11 treated with estradiol and/or IL-1 and TNFα. Nuclear extracts of +/+LDA11 cells pretreated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated or yeast extract containing recombinantly expressed human wild-type ER_{gly} were incubated with the vitellogenin ERE as probe in the absence or presence of anti-ER antibody. Complexes formed were analyzed by EMSA.

The complexes detected are unrelated to the ER since they were not significantly affected by anti-ER antibody. Controls using ER containing extracts obtained from a yeast expression system gave rise to two slowly migrating complexes that were specifically shifted with the anti-ER antibody. These data suggest

that ER is present in +/+LDA11 cells but at concentrations too low to be detected by EMSA.

DNA-binding activity of nuclear extracts to the IL-6 promoter

To study the molecular mechanism of IL-6 induction, and its repression by estrogen, nuclear extracts from +/+LDA11 cells were analyzed for DNA-binding activity to the IL-6 promoter region that mediated cytokine induction and estrogen suppression in the cotransfection experiments. Since this DNA fragment showed a high background binding with nuclear extracts the most proximal region containing the TATA box was removed leaving a fragment from -225 to -52 upstream of the transcriptional start site. This region of the promoter contains consensus binding sites for several transcription factors including a core sequence of the cAMP response element (CRE), a binding site for the leucine zipper protein NF-IL6, and a NFκB site (Isshiki, H., S. Akira, O. Tanabe, T. Nakajima, T. Shimamoto, T. Hirano, and T. Kishimoto (1990) "Constitutive and interleukin-1 (IL-1)-inducible factors interact with the IL-1-responsive element in the IL-6 gene," Mol. Cell Biol. 10:2757-2764).

Binding of NF-IL6 and NFκB-like proteins to these sites has been demonstrated (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Libermann, T.A. and D. Baltimore (1990) "Activation of interleukin-6 gene expression through the NF-kappa B transcription factor," Mol. Cell Biol. 10:2327-2334). This fragment was incubated with nuclear extracts from +/+LDA11 cells that had been treated with IL-1 and TNFα for various times. +/+LDA11 cells were pretreated with 10 nM estradiol as indicated.

After 24 h the cells were induced with TNFα and IL-1 (1 nM each) for various periods of time. Induction was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -225 to -52 IL-6 promoter fragment as probe (Figure 1a).

Complexes formed were analyzed by EMSA. After treatment with the cytokines an inducible complex was observed. The intensity of the complex was maximal already after 10 min treatment with IL-1 and TNFα and decreased gradually over time. After 2 hours of induction the intensity of the complex was significantly reduced.

Pretreatment of the cells with estradiol had no effect on the binding capacity of extracts from uninduced cells. However, estradiol pretreatment resulted in a marked increase of the induced complex with induction intervals from 10 min to 40 min but only a slight effect on the complex after 2 h of induction. In addition to the increased intensity, pretreatment with estradiol also caused a qualitative change, increasing the mobility of the complex.

Detecting the composition of the DNA-binding complex

To investigate the nature of the complex and the proteins potentially involved, we incubated the binding reactions with antibodies directed against several potential binding factors. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 10 min) as indicated were incubated with the -225 to -52 probe in the absence or presence of various antibodies. Complexes formed were analyzed by EMSA.

Fig. 1b shows that none of the antibodies tested affected DNA binding of extracts from uninduced cells. Neither anti-c-jun, nor anti-c-rel, nor anti-NF-IL6

antibodies had any effect on the cytokine induced complexes.

However, anti-p50 and anti-p65, antibodies directed against the two proteins in the NFκB complex, abolished formation of the complex (lanes 10-13). This was observed with extracts derived from cells treated or untreated with estradiol, over the whole period of cytokine induction (Fig. 1b depicts the results at 10 minutes after the induction). With longer exposures, a very weak complex of low mobility was detected, probably resulting from a supershift of the induced complex by anti-p50 and anti-p65.

Although ER binding activity to the vitellogenin ERE was not detectable in +/+LDA11 extracts, we tested whether the ER was involved in complex formation on the IL-6 promoter fragment. Nuclear extracts of +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated or yeast extract containing recombinantly expressed human wild-type ER were incubated with the -225 to -52 probe in the absence or presence of anti-ER antibody. Complexes formed were analyzed by EMSA.

Fig. 1c shows that independent of cytokine induction or estradiol treatment, addition of the anti-ER antibody did not significantly affect any of the complexes, induced or constitutive. In addition, when yeast extracts containing high concentrations of recombinant ER were incubated with the IL-6 promoter fragment no specific binding of the ER was detected (lanes 7 and 8). The weak bands observed are unrelated to the ER, since they were not affected by the anti-ER antibody.

The results from the antibody gel shift experiments were further supported by oligonucleotide competition studies. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 10 min) as indicated were incubated with the -225 to -52

probe in the absence or presence of a 400-fold molar excess of oligonucleotides corresponding to the regions of -82 to -47 and -172 to -131 of the human IL-6 promoter. Complexes formed were analyzed by EMSA.

The arrows in Fig. 1d indicate the complexes formed upon induction with TNFα and IL-1. Inclusion of an oligonucleotide covering the NF-IL6 site, the CRE, and an adjacent CCAAT-box of the IL-6 promoter (-172 to -131) in the binding reaction in 400-fold excess over the labeled -225 to -52 fragment did not affect any of the complexes, constitutive or cytokine-induced (lanes 4 and 7). However, an oligonucleotide covering the putative NFκB site and adjacent sequences (-82 to -47) specifically abolished the formation of the cytokine induced complexes (lanes 3 and 6).

The antibody experiments and the oligonucleotide competition studies suggested that IL-1 and TNFα specifically activated NFκB or related proteins. No binding of c-jun (AP-1), NF-IL6, c-rel, or ER was detected.

The lack of NF-IL6 binding is surprising, since induction and binding of this transcription factor in response to IL-1 has been reported for other cells (Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto (1990) "A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family," EMBO J. 9:1897-1906; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. USA 88:3715-3719). Our DNA binding experiments show that in the bone marrow derived +/+LDA11 cell IL-1 and TNFα induce the binding of NFκB or closely related proteins to the IL-6 promoter. Similar results have been obtained in different cell types (H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto,

and K. Yamamoto (1990) "Involvement of a NF-kappa B-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines," Mol. Cell Biol. 10:561-568; Zhang, Y.H., J.X. Lin, and J. Vilcek (1990) "Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence," Mol. Cell Biol. 10:3818-3823). Neither induced nor uninduced binding of several other factors with potential binding sites in the proximal IL-6 promoter fragment could be detected, including AP-1. This transcription factor is one of the paradigms for direct inhibition by intracellular receptor including GR, RAR and TR. The mechanism of AP-1 inhibition can involve protein-protein interaction and/or competition for DNA binding depending on the particular gene (Diamond, M.I., J.N. Miner, S.K. Yoshinaga, and K.R. Yamamoto (1990) "Transcription factor interactions: selectors of positive or negative regulation from a single DNA element," Science 249:1266-1272; Schüle, R., K. Umesono, D.J. Mangelsdorf, J. Bolado, J.W. Pike, and R.M. Evans (1990) "Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene," Cell 61:497-504; Yang-Yen, H.F., J.C. Chambard, Y.L. Sun, T. Smeal, T.J. Schmidt, J. Drouin, and M. Karin (1990) "Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction," Cell 62:1205-1215; Yang-Yen, H.F., X.K. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin, and M. Pfahl (1991) "Antagonism between retinoic acid receptors and AP-1: implications for tumor promotion and inflammation," New Biol. 3:1206-1219; Zhang, X.K., K.N. Wills, M. Husmann, T. Hermann, and M. Pfahl (1991) "Novel pathway for thyroid hormone receptor action through interaction with jun and fos oncogene

activities," Mol. Cell Biol. 11:6016-6025). Several reports also suggest a cross-talk between ER and AP-1, however there is no evidence for estrogen dependent inhibition of AP-1 activity (Gaub, M.P., M. Bellard, I. Scheuer, P. Chambon, and P. Sassone-Corsi (1990) "Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex," Cell 63:1267-1276; Tzukerman, M., X.K. Zhang, and M. Pfahl (1991) "Inhibition of estrogen receptor activity by the tumor promoter 12-O-tetradeconylphorbol-13-acetate: a molecular analysis," Mol. Endocrinol. 5:1983-1992). Taken together, it is highly unlikely that AP-1 plays a role in the negative regulation of IL-6 expression by estrogen.

Example 3. Screening agents that differentially affect distinct complexes with the IL-6 promoter

Distinctive complexes with the IL-6 promoter

Treatment of +/+LDA11 cells with IL-1 and TNFα induced binding of NFκB or related proteins to the IL-6 promoter. Since pretreatment with estradiol not only increased the intensity but also the mobility of the complexes, we investigated the binding of +/+LDA11 nuclear extracts to the oligonucleotide covering the putative NFκB site (-82 to -47).

+/+LDA11 cells were pretreated with 10 nM estradiol as indicated. After 24 h the cells were induced with TNFα and IL-1 (1 nM each) for various periods of time. Induction was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -82 to -47 IL-6 promoter fragment as probe. Fig. 2a shows that extracts from cells treated with IL-1 and TNFα exhibited 3 induced complexes (A,B,C) when compared with extracts from untreated cells.

Over the course of induction (10-120 min) in particular the fastest migrating complex (C) decreased in intensity. Interestingly, estradiol pretreatment reduced the intensity of the slowest migrating complex (A) while strongly increasing the intensity of the fastest band (C). This corresponds to the pattern obtained with the -225 to -52 fragment where complexes seemed to migrate faster with estradiol treatment (Fig. 3).

It is likely that with both fragments analogous complexes were formed; however, only with the shorter oligonucleotide were they completely resolved. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated were incubated with the -82 to -47 probe in the absence or presence of a 100-fold molar excess of oligonucleotides corresponding to the regions of -82 to -47 and -172 to -131 of the human IL-6 promoter or the vitellogenin ERE. Complexes formed were analyzed by EMSA.

All three induced complexes (A,B,C) were specific since their formation was abolished by inclusion of a 100-fold excess of the unlabeled probe (Fig. 2b, lanes 6 and 10), while the same molar excess of the NF-IL6 oligonucleotide (-172 to -131) or the vitellogenin ERE had no effect (Fig. 2b, lanes 7, 8, 11, and 12).

Fig. 2c shows that when binding of the extracts to the oligonucleotide covering the NF-IL6 site (-172 to -131) was investigated, several complexes were detected. Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 40 min) as indicated were incubated with the -172 to -131 probe in the absence or presence of a 100-fold molar excess the unlabeled oligonucleotide. Complexes formed were analyzed by EMSA. The arrows in Fig. 2c indicate

the complexes A, B, and C formed upon induction with TNFα and IL-1.

Two of the complexes were specific, since they could be competed with an excess of the unlabeled oligonucleotide (lanes 2, 4, and 6). However, all of the complexes were formed constitutively, independent of cytokine induction or estradiol treatment, suggesting that they were unrelated to the regulation of IL-6 expression by IL-1, TNFα, and estrogen.

Screening for compounds that affect the formation of distinct complexes

In more detailed studies we analyzed the effects of other compounds on the formation of complexes A, B, and C (Fig. 3). +/+LDA11 cells were pretreated with cycloheximide (CHX) or the kinase inhibitor H7 for 5 min or with estradiol (10 nM) and/or ICI 164,384 (100 nM) for 24 h or 60 min before induction with TNFα and IL-1 (1nM each for 30 min). Treatment was stopped by cell lysis and nuclear extracts were analyzed by EMSA using the -82 to -47 fragment as probe. Arrows indicate the induced complexes A, B, and C.

Pretreatment of the cells with the protein synthesis inhibitor cycloheximide before addition of cytokines did not interfere with complex formation (lane 8). This is consistent with the fast induction of binding and has been shown before for the activation of NFκB (Henkel, T., T. Machleidt, I. Alkalay, M. Kronke, Y. Ben-Neriah, and P.A. Baeuerle (1993) "Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B," Nature 365:182-185; Sen, R. and D. Baltimore (1986) "Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism," Cell 47:921-928). It has been reported that cycloheximide treatment activates NFκB binding (Sen, R. and D. Baltimore (1986)

"Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism," Cell 47:921-928; Zhang, Y.H., J.X. Lin, and J. Vilcek (1990) "Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence," Mol. Cell Biol. 10:3818-3823). However, in +/+LDA11 cells we did not observe any induction by cycloheximide (lane 7). Additionally, pretreatment with H-7, a potent protein kinase C (PKC) inhibitor (Kawamoto, S. and H. Hidaka (1984) "1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) is a selective inhibitor of protein kinase C in rabbit platelets," Biochem. Biophys. Res. Commun. 125:258-264), did not interfere with induction of complex formation by IL-1 and TNFα (lane 10). This suggests that induction of complexes A, B, and C is mediated through a PKC-independent pathway and is consistent with the finding that IL-1 and TNFα activate NFκB and induce IL-6 independently of PKC (11,55,99).

Activation of NFκB by phorbol esters is probably mediated by PKC (Baeuerle, P.A. and D. Baltimore (1988) "I kappa B: a specific inhibitor of the NF-kappa B transcription factor," Science 242:540-546). However, treatment of +/+LDA11 cells with 12-O-tetradeconylphorbol-13-acetate (TPA) did not significantly induce production of IL-6, nor did it induce complex formation with the -82 to -47 fragment.

The estradiol effect, i.e. decreasing complex A and increasing complex C (Fig. 3, compare lanes 2 and 3), was not seen with a short estradiol pretreatment (60 min) before induction (lane 6). In addition, the pure anti-estrogen ICI 164,384 (Wakeling, A.E. and J. Bowler (1988) "Biology and mode of action of pure antioestrogens," J. Steroid Biochem. 30:141-147) did not affect the complex pattern (lane 4). However, when ICI

164,384 was added in combination with estradiol it prevented the effect mediated by the estrogen (lane 5).

Since ICI 164,384 acts as an antagonist via binding to the ER, these results further support the hypothesis that the effects of estradiol on the induced complexes are receptor mediated. However, the mechanism of estrogen action is probably indirect, as indicated by the lack of response to short term estradiol treatment.

<u>Screening for agents that affect binding characteristics of the proteins in distinct complexes</u>

To investigate the binding characteristics of the proteins in complexes A, B, and C with the NFκB oligonucleotide, methylation interference experiments were carried out (Fig. 4). Nuclear +/+LDA11 extracts from cells induced with TNFα and IL-1 (1nM each) were incubated with -82 to -47 probe that had been labeled either on the upper or the lower strand and subjected to limited DMS-methylation. After preparative EMSA, DNA from complexes A, B, and C and from the unretarded probe (F) was isolated, cleaved with piperidine, and electrophoresed on a 12% denaturing gel. The sequence corresponding to the NFκB consensus site is shown boxed, a cryptic AP-1 site is shaded.

On both strands N-7-methylation of the guanine bases within the NFκB site (-73 to -63, boxed) interfered with complex formation, while methylation of guanines flanking the consensus site had no observable effect. The interference pattern for all three complexes (A,B,C) was identical.

The observation that methylation of guanines just outside of the NFκB site (-75, -60, -58) did not affect the formation of even the largest complex (A) suggests that in all three complexes DNA contacts are made within the same core region. In addition, the lack of DNA binding interference with methylation of guanines -60, -

58, and -56 strongly argues against any cytokine induced binding of factors to the nonconsensus (TGAGTCT, shaded) AP-1 site (Tanabe, O., S. Akira, T. Kamiya, G.G. Wong, T. Hirano, and T. Kishimoto (1988) "Genomic structure of the murine IL-6 gene. High degree conservation of potential regulatory sequences between mouse and human," J. Immunol. 141:3875-3881) in this region (-61 to -55).

Our studies show that estrogen affects the formation of complexes with the IL-6 promoter that involve NFκB p50 and p65 or very closely related proteins. Treatment of +/+LDA11 cells with IL-1 and TNFα specifically induced the formation of at least three distinct complexes with the NFκB consensus site in the IL-6 promoter. Although of various size, in all three complexes the DNA contacts are restricted to the core sequence of the NFκB site. The corresponding core sequence of other NFκB elements is protected by p50 and p65 (Baldwin, A.S., Jr. and P.A. Sharp (1988) "Two transcription factors, NF-kappa B and H2TF1, interact with a single regulatory sequence in the class I major histocompatibility complex promoter," Proc. Natl. Acad. Sci. USA 85:723-727; Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M.B. Urban, P. Kourilsky, P.A. Baeuerle, and A. Israel (1990) "The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product," Cell 62:1007-1018; Sen, R. and D. Baltimore (1986) "Multiple nuclear factors interact with the immunoglobulin enhancer sequences," Cell 46:705-716), both of which have been shown to directly interact with DNA (Nolan, G. P., S. Ghosh, H.C. Liou, P. Tempst, and D. Baltimore (1991) "DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide," Cell 64:961-969; Urban, M.B., R. Schreck, and P.A. Baeuerle (1991) "NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit," EMBO J.

10:1817-1825). C-rel homodimers and heterodimers with p50 have been shown to bind the NFκB site in the IL-6 promoter (Nakayama, K., H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1992) "A lymphoid cell-specific nuclear factor containing c-Rel-like proteins preferentially interacts with interleukin-6 kappa B-related motifs whose activities are repressed in lymphoid cells," Mol. Cell Biol. 12:1736-1746).

In addition, this study showed that in lymphoid cells c-rel or an immunologically related factor is a component of a larger complex that binds the NFκB site in the IL-6 promoter and functions as a constitutive repressor. In +/+LDA11 cells, we could not detect any c-rel specific binding activity. A number of other NFκB unrelated proteins have been shown to bind to NFκB consensus sites. Those include aA-CRYBP1 (Nakamura, T., D.M. Donovan, K. Hamada, C.M. Sax, B. Norman, J.R. Flanagan, K. Ozato, H. Westphal, and J. Piatigorsky (1990) "Regulation of the mouse alpha A-crystallin gene: isolation of a cDNA encoding a protein that binds to a cis sequence motif shared with the major histocompatibility complex class I gene and other genes," Mol. Cell Biol. 10:3700-3708), MBP-1/PRDII-BFI (Baldwin, A.S., Jr., K.P. LeClair, H. Singh, and P.A. Sharp (1990) "A large protein containing zinc finger domains binds to related sequence elements in the enhancers of the class I major histocompatibility complex and kappa immunoglobulin genes," Mol. Cell Biol. 10:1406-1414; Fan, C.M. and T. Maniatis (1990) "A DNA-binding protein containing two widely separated zinc finger motifs that recognize the same DNA sequence," Genes Dev. 4:29-42), and AGIE-BP1 (Ron, D., A.R. Brasier, and J.F. Habener (1991) "Angiotensinogen gene-inducible enhancer-binding protein 1, a member of a new family of large nuclear proteins that recognize nuclear

factor kappa B-binding sites through a zinc finger motif," Mol. Cell Biol. 11:2887-2895).

It has been shown that C/EBP-like proteins attenuate NFκB mediated transactivation of the angiotensinogen gene acute-phase response element (Brasier, A.R., D. Ron, J.E. Tate, and J.F. Habener (1990) "A family of constitutive C/EBP-like DNA binding proteins attenuate the IL-1 alpha induced, NF kappa B mediated trans-activation of the angiotensinogen gene acute-phase response element," EMBO J. 9:3933-3944). Currently, we cannot exclude that those proteins or others are part of the observed complexes or are involved in the inhibition of IL-6 expression by estrogen. A recent study suggested that in uterine cells, estradiol induced complex formation with an NFκB element (Shyamala, G. and M.C. Guiot (1992) "Activation of kappa B-specific proteins by estradiol," Proc. Natl. Acad. Sci. USA 89:10628-10632). The induced complex did not contain p50 or p65 and therefore may represent other factors.

Example 4. Screening for agents that affects the binding of p65 to IL-6 promoter

Analyzing composition of the complexes formed with the NFκB site

As with the larger promoter fragment, we analyzed the nature of the complexes formed with the NFκB oligonucleotide (-82 to -47) by antibody shift experiments (Fig. 5a). Nuclear extract from +/+LDA11 cells treated with estradiol (10 nM) and TNFα and IL-1 (1nM each for 10 min) as indicated were incubated with the -82 to -47 probe in the absence or presence of various antibodies. Complexes formed were analyzed by EMSA.

We observed strong effects on the induced complexes when anti-p50 or anti-p65 (lanes 17-20) were included in

the binding reactions. Interestingly, anti-p50 specifically abolished the formation of complexes B and C, seemed to leave complex A unaffected, and caused the appearance of a single supershifted band (S1). Anti-p65, however, inhibited the formation of all three induced complexes and produced two supershifted bands (S1, S2). This suggested that p65 or an immunologically closely related protein is part of all three induced complexes, while p50 or a related protein is only present in complexes B and C.

It has been reported that recombinantly expressed c-rel binds to the NFκB site in the IL-6 promoter as heterodimer with p50 and, with particular high affinity, as homodimer (Nakayama, K., H. Shimizu, K. Mitomo, T. Watanabe, S. Okamoto, and K. Yamamoto (1992) "A lymphoid cell-specific nuclear factor containing c-Rel-like proteins preferentially interacts with interleukin-6 kappa B-related motifs whose activities are repressed in lymphoid cells," Mol. Cell Biol. 12:1736-1746). When anti-c-rel was included in the binding reactions with the +/+LDA11 extracts the antibody did not inhibit any of the induced complexes. On longer exposures a weak supershifted complex was detectable. This complex migrated at the same position as the supershift observed with anti-p50 suggesting that it did not contain the larger c-rel protein. Since the peptide used to raise the anti-c-rel antibody has a 56% homology to the analogous p50 sequence (Ghosh, S., A.M. Gifford, L.R. Riviere, P. Tempst, G.P. Nolan, and D. Baltimore (1990) "Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal," Cell 62:1019-1029; Inoue, J., L.D. Kerr, L.J. Ransone, E. Bengal, T. Hunter, and I.M. Verma (1991) "c-rel activates but v-rel suppresses transcription from kappa B sites," Proc. Natl. Acad. Sci. USA 88:3715-3719), it is likely that this weak band is the result of a cross-reactivity and unrelated to c-

rel. As with the larger promoter fragment, anti-c-jun did not affect complex formation (lanes 13 and 14). Results shown depict the 10 min induction time point and are essentially identical with longer cytokine treatments.

None of the antibodies tested had a marked effect on complex formation with the uninduced extracts. Only anti-p50 produced a very weak supershifted complex migrating at the S1 position as observed with the induced extracts (only visible on longer exposures). This weak binding activity, only detectable when supershifted, could either result from a cytosolic contamination or represent basal activation under the culture conditions.

In additional EMSA experiments we included a purified preparation of recombinant p50 as well as the yeast expressed ER (Fig 5b). Nuclear +/+LDA11 extracts from cells pretreated with estradiol (10 nM) and induced with TNFα and IL-1 (1nM each for 30 min) as indicated as well as purified human p50 protein and yeast extract containing recombinantly expressed human ER were incubated with -82 to -47 probe in the absence or presence of various antibodies. Complexes formed were analyzed by EMSA. Arrows indicate complexes A, B, and C induced by cytokine treatment and the complexes S1 and S2 resulting from the antibody supershifts.

As expected, neither did yeast expressed ER bind to the NFκB -82 to -47 fragment (lanes 5, 9, 13, and 17) nor was ER involved in the formation of the induced complexes as indicated by the lack of any anti-ER antibody effect (lanes 14-17). Purified p50 bound to this fragment and was specifically supershifted by anti-p50 but not by anti-p65 (compare lanes 4, 8, and 12).

Surprisingly, the complex formed with purified recombinant p50 migrated slower than complexes B and C. Using lower concentrations of purified p50 did not

affect the migration, suggesting that the band represented p50 homodimers and not higher order complexes (Duckett, C.S., N.D. Perkins, T.F. Kowalik, R.M. Schmid, E.S. Huang, A.S. Baldwin, Jr., and G.J. Nabel (1993) "Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3)," Mol. Cell Biol. 13:1315-1322). However, the antibody shift experiments suggested that both NFκB proteins, p50 and p65, are part of complexes B and C (lanes 6, 7, 11, and 12) and consequently both complexes should migrate slower than p50 homodimers (Urban, M.B., R. Schreck, and P.A. Baeuerle (1991) "NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit," EMBO J. 10:1817-1825).

It is possible that the proteins in complexes B and C are only immunologically related to p50 and p65 but actually of smaller size. Speculations that the p50 homologue p49 is part of the induced complexes and is responsible for the faster migration could not be confirmed. Although purified p49 bound the -82 to -47 IL-6 fragment and strongly cross-reacted with the anti-p50 antibody, the complex formed migrated even more slowly than the p50 complex. This corresponds to results obtained with other NFκB binding sites (Duckett, C.S., N.D. Perkins, T.F. Kowalik, R.M. Schmid, E.S. Huang, A.S. Baldwin, Jr., and G.J. Nabel (1993) "Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3)," Mol. Cell Biol. 13:1315-1322).

However, the finding that the anti-p50 antibody strongly cross-reacts with p49 indicates that the antibodies used may detect other NFκB related proteins in the complexes. Alternatively, the migration of complexes B and C could be higher than the migration

observed with recombinant p50 due to conformational differences resulting from post-translational modification. This would correlate with the observation that the inclusion of anti-p50, abolishing complexes B and C, produced a supershifted complex (S1) migrating more slowly than the supershifted complex obtained with recombinant p50 (Fig. 5b, lanes 6-8).

A closer inspection of the band shift results indicated that anti-p50 also affected complex A. As discussed before, treatment with estradiol not only increased the intensity of complex C but also decreased the intensity of complex A (lanes 2 and 3). We consistently observed that inclusion of anti-p50 had a very similar effect: the antibody specifically decreased the intensity of complex A formed with the extracts from cells not treated with estradiol, resulting in equal intensity of this band using extracts from estradiol treated or untreated cells (Fig. 5b, compare lanes 2 and 7). These results suggest that band A induced by IL-1 and TNFα in the absence of estradiol is composed of two different unresolved complexes, one (A1) that is also induced in the presence of estradiol and does not contain p50, and another complex (A2) containing p50 (or an immunologically related protein).

Treatment with estradiol may increase the intensity of complex C at the expense of complex A2. If complex A2 represents a transcriptionally more active state, this could explain the inhibitory effect of estradiol on IL-6 expression. Recently it has been shown that the TATA binding protein (TBP or TFIIDt) directly interacts with NFκB (Kerr, L.D., L.J. Ransone, P. Wamsley, M.J. Schmitt, T.G. Boyer, Q. Zhou, A.J. Berk, and I.M. Verma (1993) "Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B," Nature 365:412-419). Therefore, we were interested if TBP was involved in the formation of the

induced complexes. However, using an anti-TBP antibody we could not detect any participation of TBP in complexes A, B, or C.

Our antibody gel shift experiments suggested that p65 is a component of all three observed complexes. This particular protein is the NFκB component containing the transactivation domain (Schmitz, M.L. and P.A. Baeuerle (1991) "The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B," EMBO J. 10:3805-3817). Within the different complexes the transactivation function may be differentially active. The antibody shift experiments suggest that estradiol diminishes the A2 complex while increasing complex C. The slow migrating A2 complex may contain other factor(s) involved in the transactivation process. The TATA-binding protein TBP, part of the TFIID complex has been reported to interact strongly with c-rel and p65, but not with p50 or p49 (Kerr, L.D., L.J. Ransone, P. Wamsley, M.J. Schmitt, T.G. Boyer, Q. Zhou, A.J. Berk, and I.M. Verma (1993) "Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptional activation by NF-kappa B," Nature 365:412-419). However, using a TBP-specific antibody we could not detect TBP as part of any of the complexes formed with the NFκB site in the IL-6 promoter.

Example 5. Efficacy-testing of Putative Cytokine Modulators

Methods for testing the efficacy of putative cytokine modulators are provided. Each candidate compound is tested for its efficacy in modulating cytokine expression in cell lines, in animal models, and in controlled clinical studies using methods known to those skilled in the art and approved by the Food and Drug Administration, such as, but not limited to, those

promulgated in The Federal Register 47 (no. 56): 12558-12564, March 23, 1982.

Example 6. Toxicity-testing of Putative Cytokine Modulators

Methods are provided for determining whether an agent active in any of the methods listed above has little or no effect on healthy cells. Such agents are then formulated in a pharmaceutically acceptable buffer or in buffers useful for standard animal tests.

By "pharmaceutically acceptable buffer" is meant any buffer which can be used in a pharmaceutical composition prepared for storage and subsequent administration, which comprise a pharmaceutically effective amount of an agent as described herein in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

A. Additional screens for Toxicity: Method 1

Agents identified as having cytokine modulating activity are assessed for toxicity to cultured human cells. This assessment is based on the ability of living cells to reduce 2,3,-bis[2-methoxy-4-nitro-5-sulphonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide] otherwise referred to as XTT (Paull et al., J. Heterocyl. Chem. 25:763-767 (1987); Weislow et al., (1989), J. Natl. Canc. Inst. 81:577). Viable mammalian cells are capable of reductive cleavage of an N-N bond

in the tetrazole ring of XTT to form XTT formazan. Dead cells or cells with impaired energy metabolism are incapable of this cleavage reaction. The extent of the cleavage is directly proportional to the number of living cells tested. Cells from a human cell line such as HeLa cells are seeded at 10^3 per well in 0.1 ml of cell culture medium (Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum) in the wells of a 96 well microtiter plate. Cells are allowed to adhere to the plate by culture at 37° C in an atmosphere of 95% air, 5% CO_2. After overnight culture, solutions of test substances are added in duplicate to wells at concentrations that represent eight half-decade log dilutions. In parallel, the solvent used to dissolve the test substance is added in duplicate to other wells. The culture of the cells is continued for a period of time, typically 24 hours. At the end of that time, a solution of XTT and a coupler (methylphenazonium sulfate) is added to each of the test wells and the incubation is continued for an additional 4 hours before the optical density in each of the wells is determined at 450 nm in an automated plate reader. Substances that kill mammalian cells, or impair their energy metabolism, or slow their growth are detected by a reduction in the optical density at 450 nm in a well as compared to a well which received no test substance.

B. Additional screens for Toxicity: Method 2

Cytokine modulators are tested for cytotoxic effects on cultured human cell lines using incorporation of ^{35}S methionine into protein as an indicator of cell viability. HeLa cells are grown in 96 well plates in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum and 50μg/ml penicillin and streptomycin. Cells are initially seeded at 10^3 cells/well, 0.1 ml/well. Cells are grown for 48 hrs without exposure to the cytokine modulator, then medium

is removed and varying dilutions of the cytokine modulator prepared in complete medium are added to each well, with control wells receiving no cytokine modulator. Cells are incubated for an additional 48-72 hrs. Medium is changed every 24 hrs and replaced with fresh medium containing the same concentration of the cytokine modulators. Medium is then removed and replaced with complete medium without antifungal. Cells are incubated for 24 hr in the absence of cytokine modulator, then viability is estimated by the incorporation of ^{35}S into protein. Medium is removed, replaced with complete medium without methionine, and incubated for 30 min. Medium is again removed, and replaced with complete medium without methionine but containing 0.1 μCi/ml ^{35}S methionine. Cells are incubated for 3 hrs. Wells are washed 3 times in PBS, then cells are permeabilized by adding 100% methanol for 10 min. Ice cold 10% trichloroacetic acid (TCA) is added to fill wells; plates are incubated on ice for 5 min. This TCA wash is repeated two more times. Wells are again washed in methanol, then air dried. 50μl of scintillation cocktail are added to each well and dried onto the wells by centrifugation. Plates are used to expose X ray film. Densitometer scanning of the autoradiogram, including wells without antifungal, is used to determine the dosage at which 50% of cells are not viable (ID_{50}) (Culture of Animal Cells. A manual of basic technique. (1987). R. Ian Freshney. John Wiley & Sons, Inc., New York).

Example 7. Administration of Cytokine Modulators

The invention features novel cytokine modulators discovered by the methods described above. It also includes novel pharmaceutical compositions which include cytokine modulators discovered as described above formulated in pharmaceutically acceptable formulations.

Furthermore, the invention features a method for treating a subject inflicted with a pathological condition affected by the level of a cytokine by administering to that subject a therapeutically effective amount of a cytokine modulator. Such administration can be by any method known to those skilled in the art, for example, by topical application or by systemic administration.

By "therapeutically effective amount" is meant an amount that relieves (to some extent) one or more symptoms of the disease or condition in the patient. Additionally, by "therapeutically effective amount" is meant an amount that returns to normal, either partially or completely, physiological or biochemical parameters associated with or causative of a mycotic disease or condition. Generally, it is an amount between about 1 nmole and 1 μmole of the molecule, dependent on its EC_{50} and on the age, size, and disease associated with the patient.

Other embodiments of this invention are disclosed in the following claims.

WHAT IS CLAIMED IS

1. Method for screening for a therapeutic agent for treatment of a pathological condition affected by the level of a cytokine, comprising the steps of:

contacting a potential therapeutic agent with a system comprising an intracellular receptor, a promoter or a portion of said promoter with a rel site, and a protein that binds to said rel site on said promoter;

measuring the binding of said protein to said rel site on said promoter; wherein a reduction in the binding of said protein to the rel site on the promoter compared to the binding of said protein in the absence of said agent is an indication that said agent is potentially useful for treatment of said condition.

2. The method of claim 1, wherein said protein is a rel-like protein.

3. The method of claim 2, wherein said rel-like protein is NFκB.

4. The method of claim 1, wherein said system further comprises a ligand for said intracellular receptor.

5. The method of claim 1, wherein said condition is osteoporosis.

6. The method of claim 1, wherein said condition is rheumatoid arthritis.

7. The method of claim 1, wherein said condition is inflammation.

8. The method of claim 1, wherein said condition is psoriasis.

9. The method of claim 1, wherein said condition is Kaposi's sarcoma.

10. The method of claim 1, wherein said condition is septic shock.

11. The method of claim 1, wherein said condition is multiple myeloma.

12. The method of claim 1, wherein said intracellular receptor is a steroid receptor.

13. The method of claim 1, wherein said intracellular receptor is an estrogen receptor.

14. The method of claim 1, wherein said intracellular receptor is selected from the group consisting of retinoid acid receptors, retinoid X receptors, glucocorticoid receptor, progesterone receptors, androgen receptor, thyroid hormone receptors, and vitamin D receptor.

15. The method of claim 1, wherein said measuring comprises determining the expression level of a cytokine or an acute phase protein.

16. The method of claim 1, wherein said measuring comprises determining the expression level of a reporter gene linked to said promoter.

17. The method of claim 1, wherein said system further comprises an effector of said promoter.

18. The method of claim 17, wherein said effector is selected from the group consisting of tumor necrosis factor, interleukin-1, viruses, endotoxin, phorbol

esters, epidermal growth factor, leukemia inhibitor factor and cAMP agonists.

19. The method of claim 1, wherein said cytokine is interleukin 6.

20. The method of claim 1, wherein said cytokine is interleukin 8.

21. The method of claim 1, wherein said system is a cell.

22. The method of claim 1, wherein said system is an extract of a cell.

23. The method of claim 21, wherein said intracellular receptor is expressed from a transfected vector.

24. The method of claim 21, wherein said promoter or said portion of said promoter is transfected into said cell.

FIG. 1a.

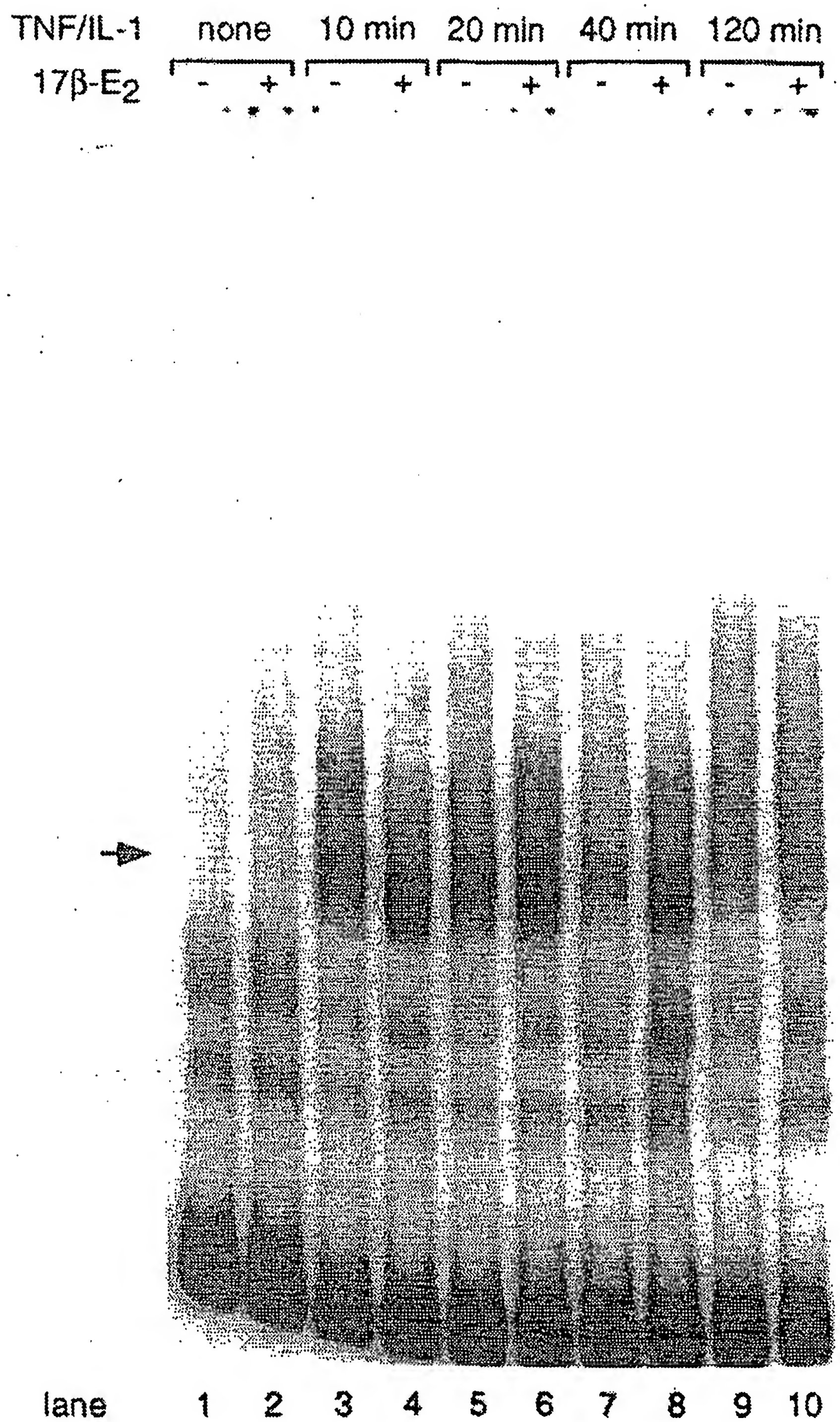
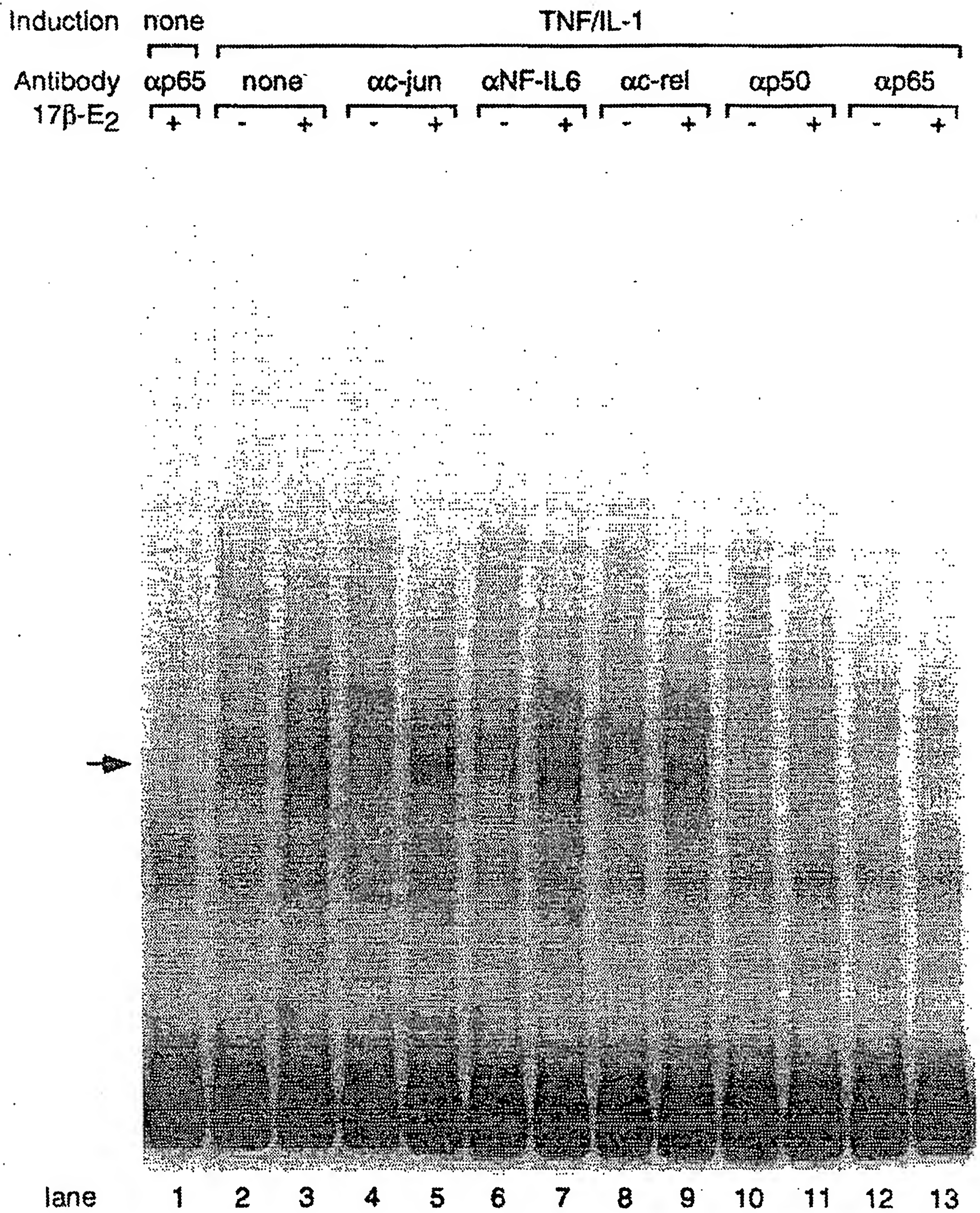

TNF/IL-1
none
10 min
20 min
40 min
120 min
17β-E2
- + - + - + - + - +
lane
1 2 3 4 5 6 7 8 9 10

FIG. 1b.



Induction
none
TNF/IL-1
Antibody
αp65
none
αc-jun
αNF-IL6
αc-rel
αp50
αp65
17β-E2
+
- +
- +
- +
- +
- +
- +
lane
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 1c.

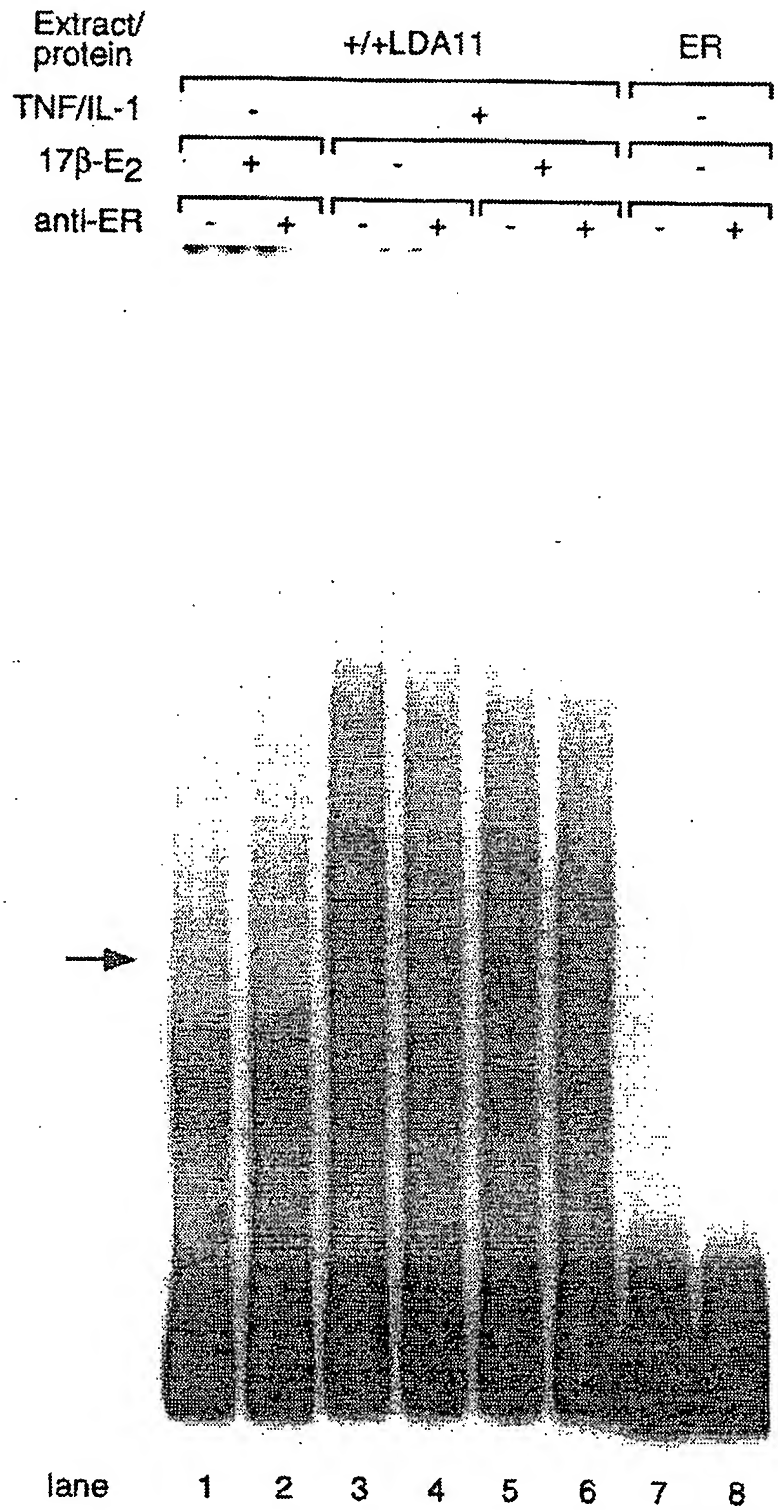

Extract/ protein
+/+LDA11
ER
TNF/IL-1
- + -
17β-E2
+ - + -
anti-ER
- + - + - + - +
lane
1 2 3 4 5 6 7 8

FIG. 1d.

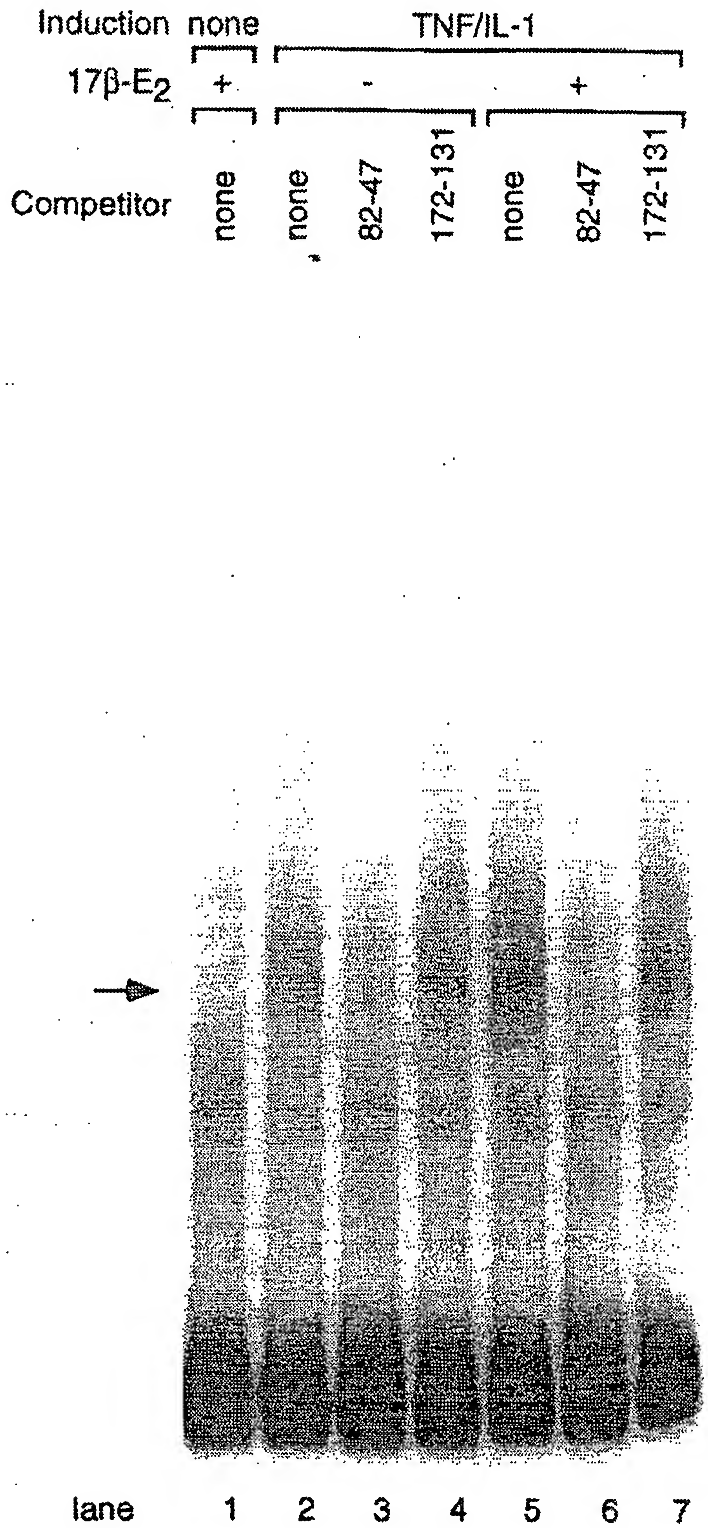

Induction
none
TNF/IL-1
17β-E2
+
-
+
Competitor
none
none
82-47
172-131
none
82-47
172-131
lane
1
2
3
4
5
6
7

FIG. 2a.

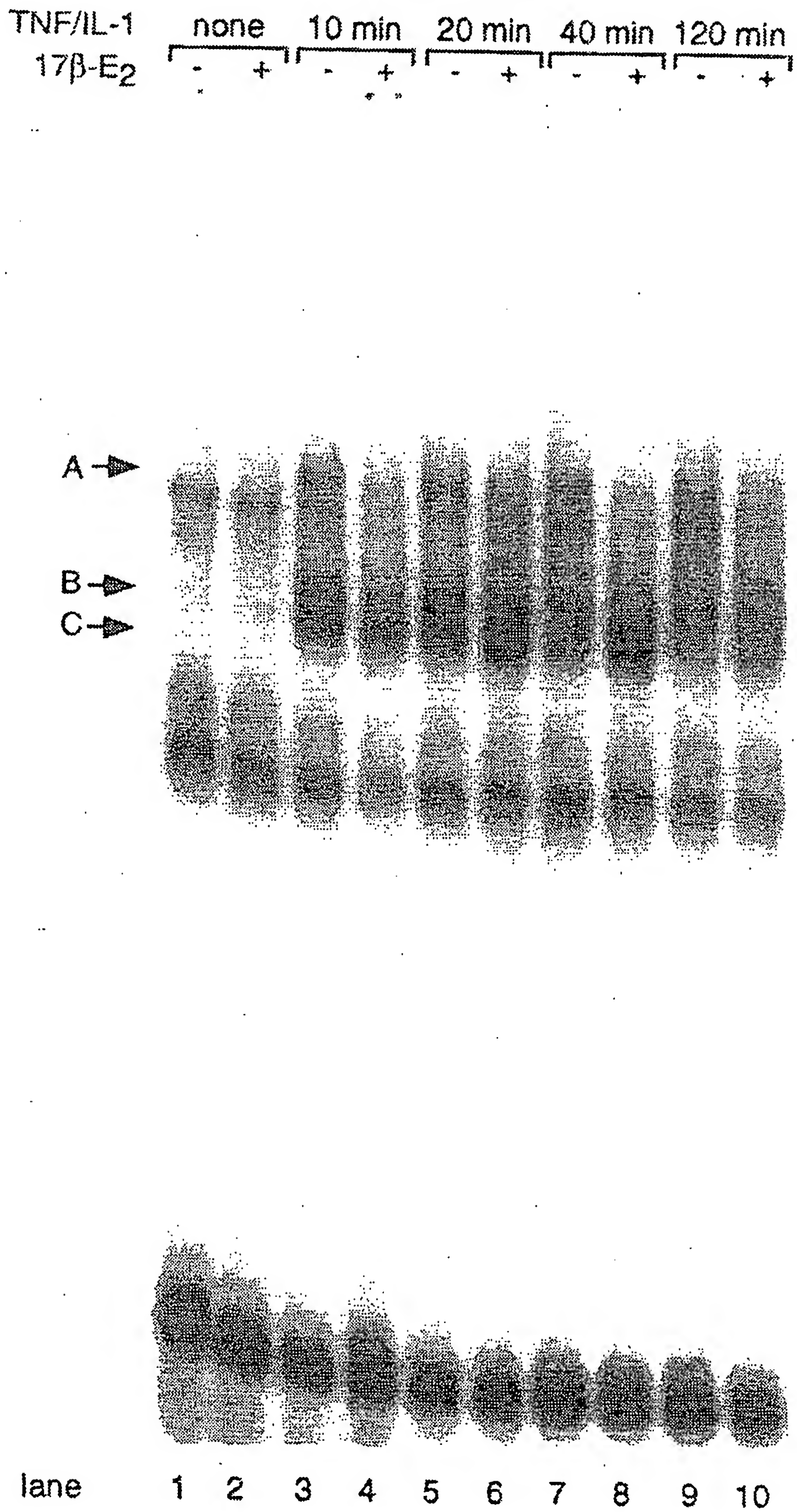

TNF/IL-1
none
10 min
20 min
40 min
120 min
17β-E2
- + - + - + - + - +
A
B
C
lane
1 2 3 4 5 6 7 8 9 10

FIG. 2b.

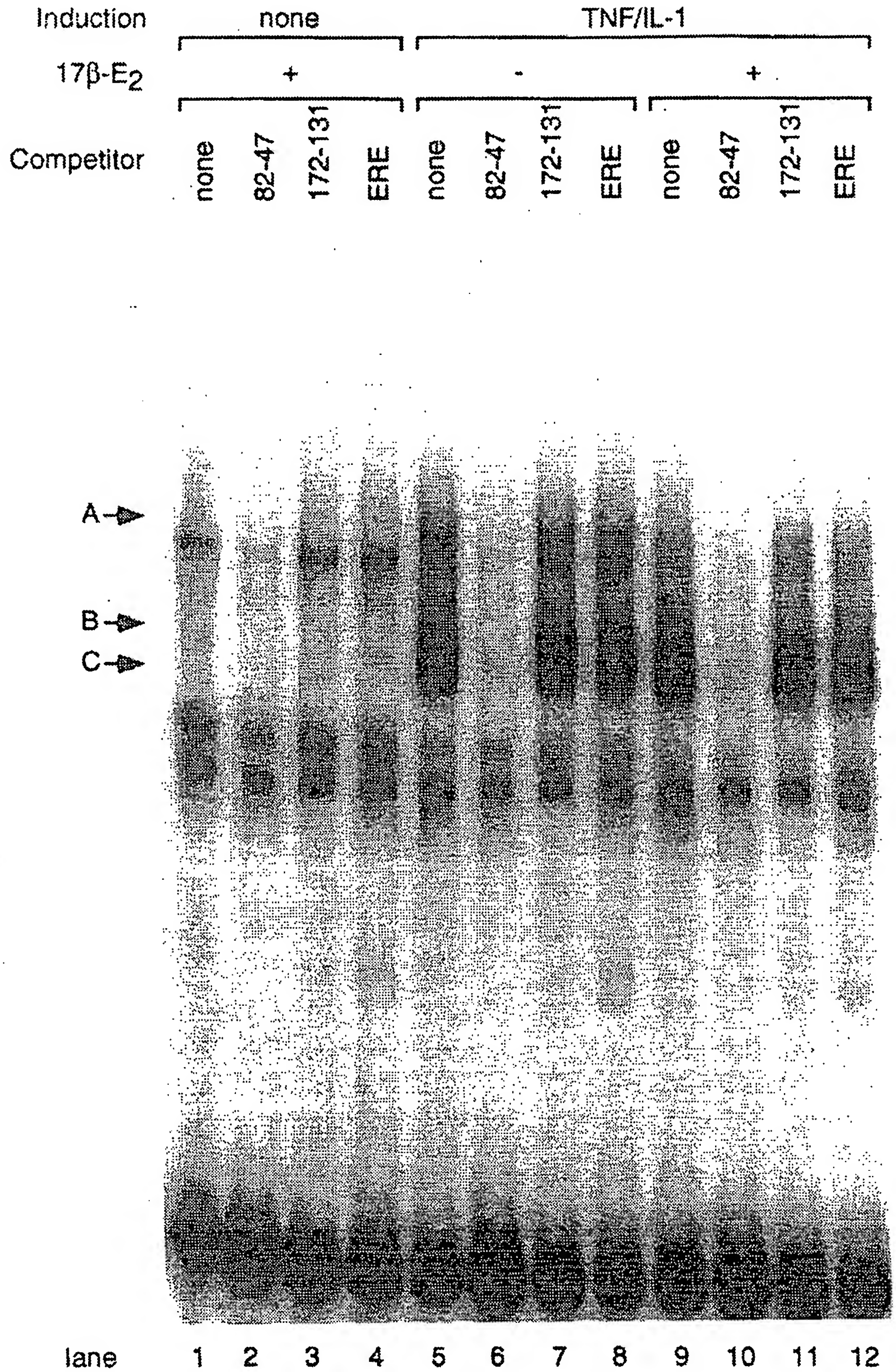

Induction
none
TNF/IL-1
17β-E2
+
-
+
Competitor
none
82-47
172-131
ERE
none
82-47
172-131
ERE
none
82-47
172-131
ERE
A
B
C
lane
1
2
3
4
5
6
7
8
9
10
11
12

FIG. 2c.

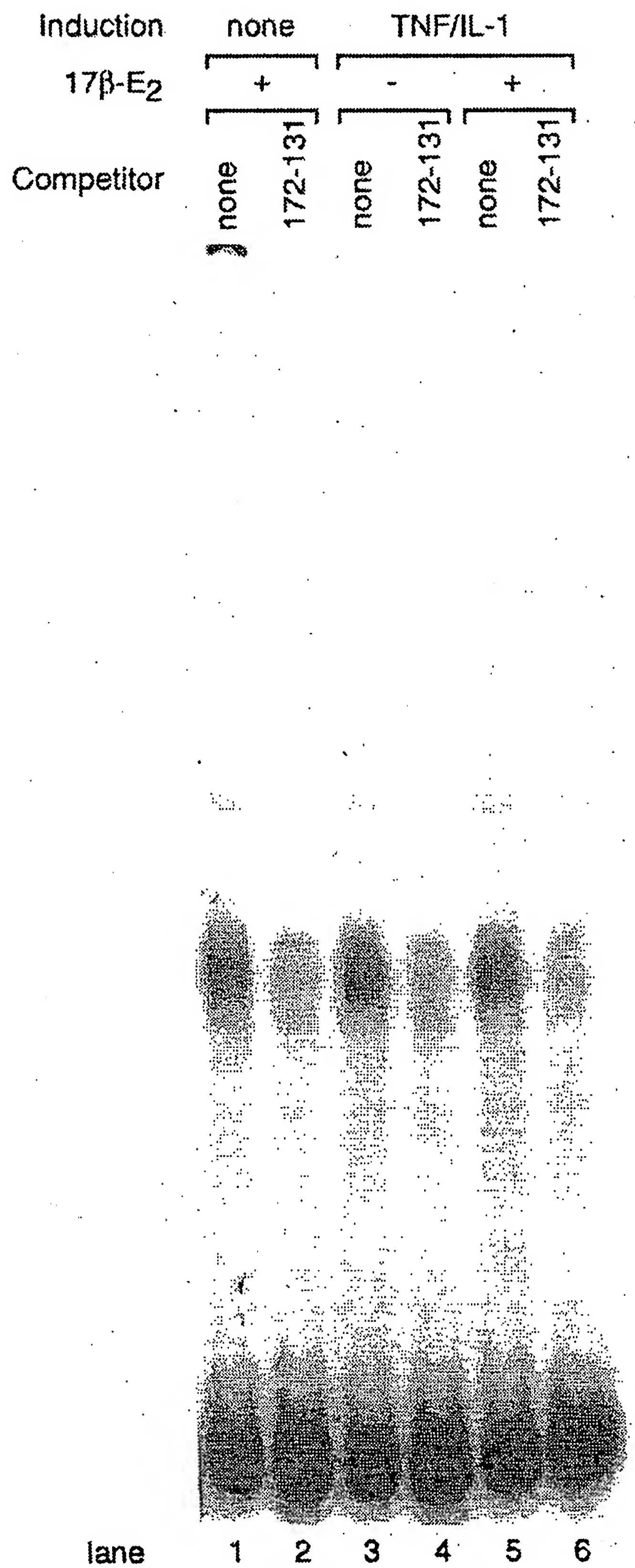

Induction
none
TNF/IL-1
17β-E2
+
-
+
Competitor
none
172-131
none
172-131
none
172-131
lane
1
2
3
4
5
6

FIG. 3.

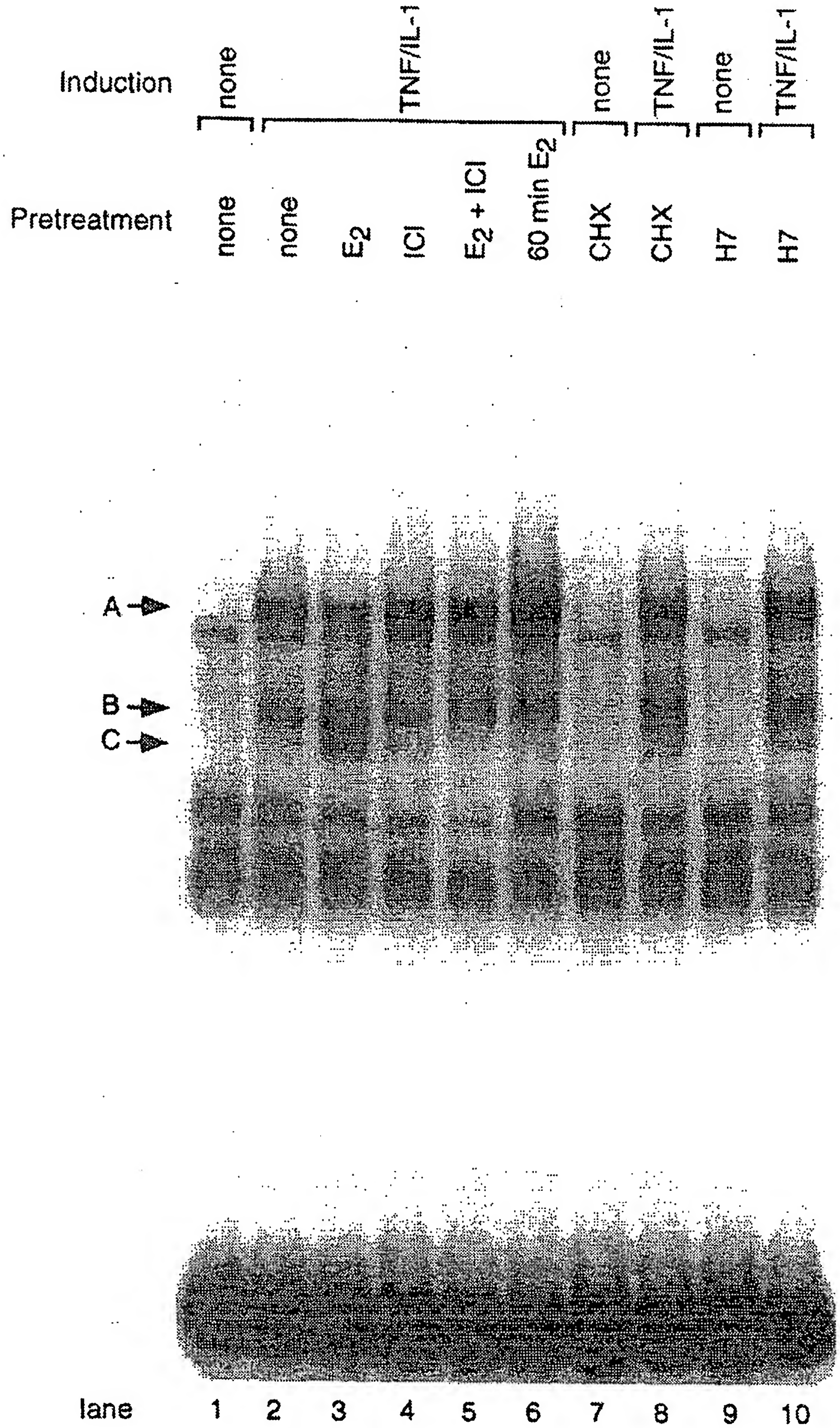

Induction
none
TNF/IL-1
none
TNF/IL-1
none
TNF/IL-1
Pretreatment
none
none
E_2
ICI
E_2 + ICI
60 min E_2
CHX
CHX
H7
H7
A
B
C
lane
1 2 3 4 5 6 7 8 9 10

FIG. 4.

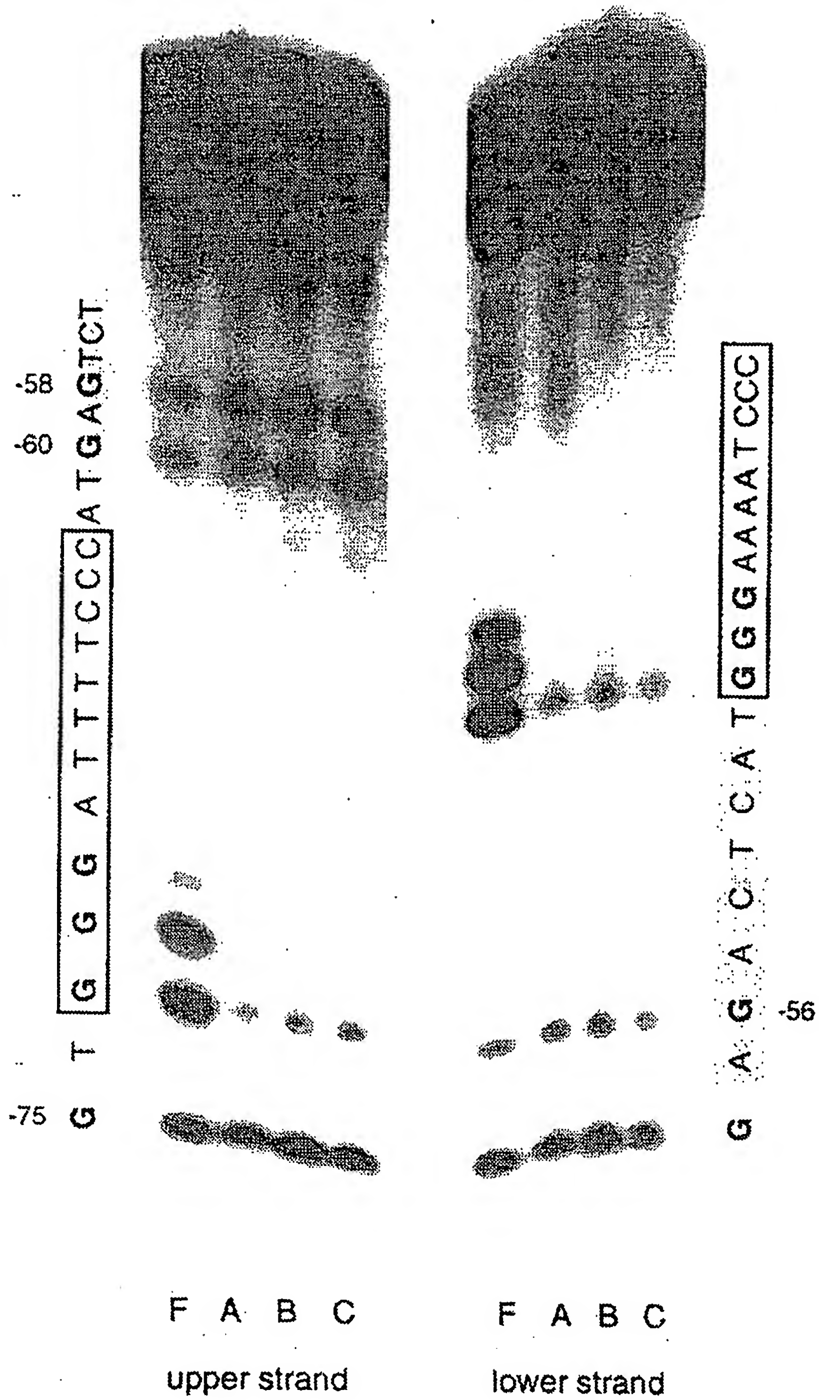

-58
-60
-75
G T G G G A T T T C C C A T G A G T C T
T G G G A A A A T C C C
G A G A C T C A T
-56
G
F A B C
upper strand
F A B C
lower strand

FIG. 5a.

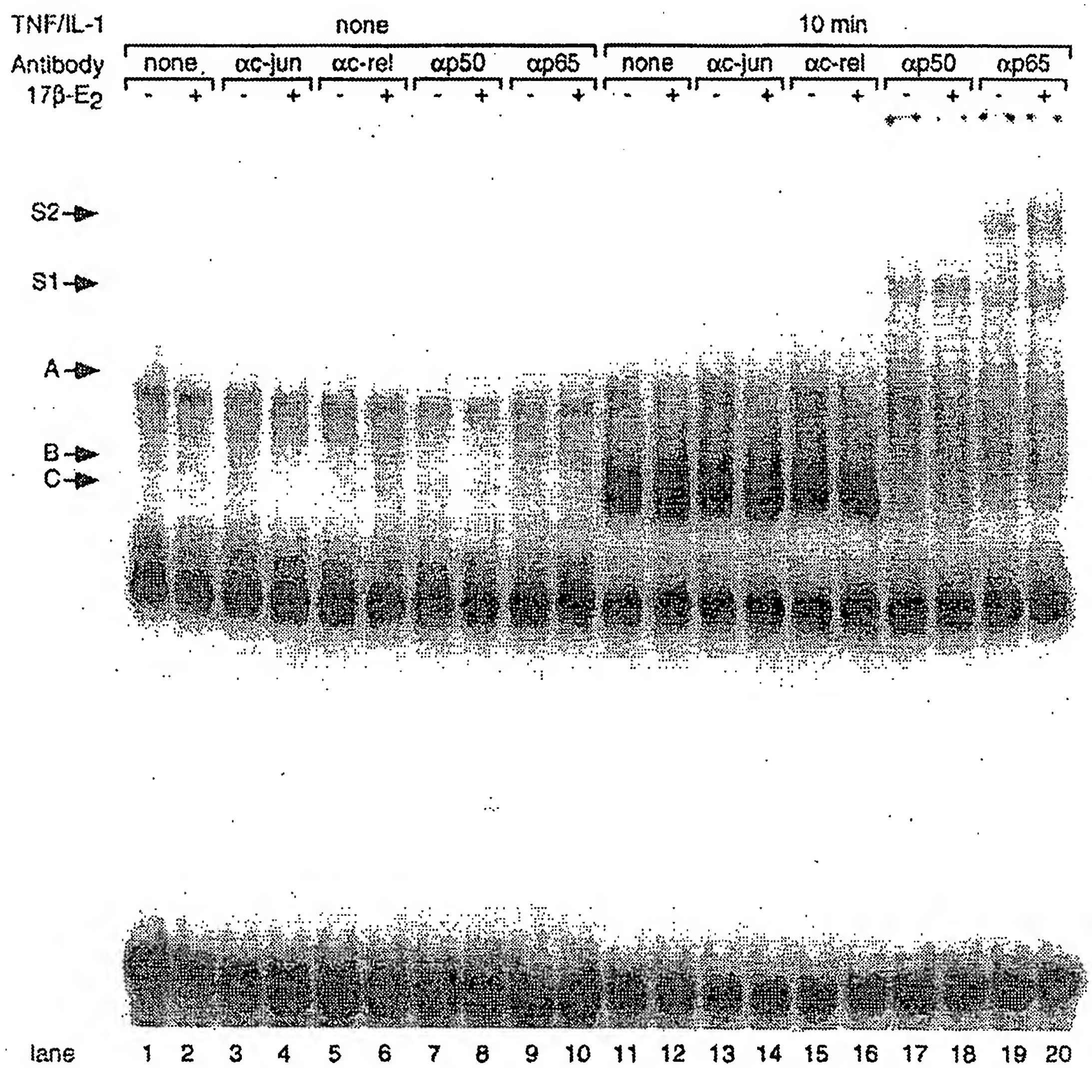
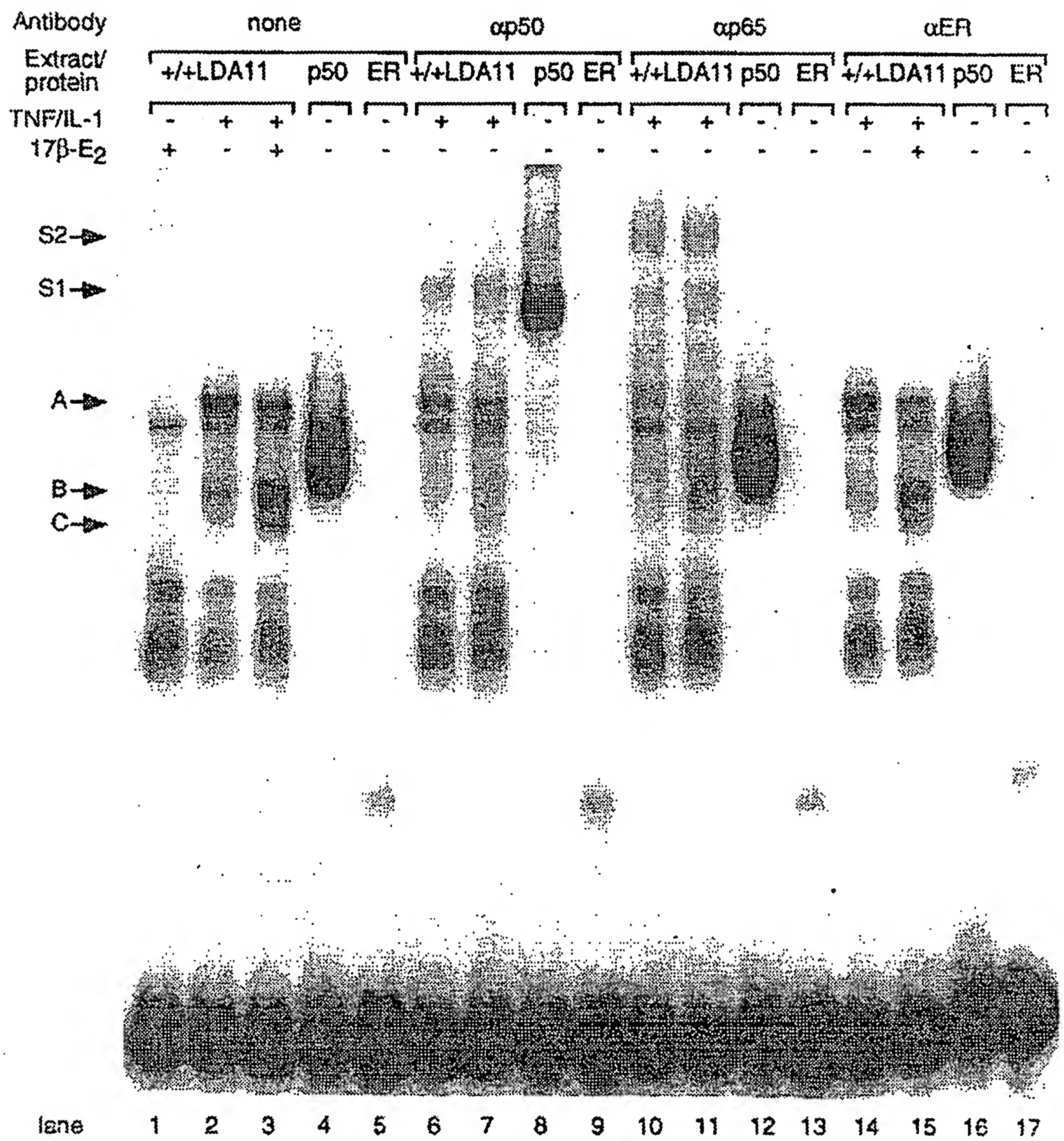

TNF/IL-1
none
10 min
Antibody
none
αc-jun
αc-rel
αp50
αp65
none
αc-jun
αc-rel
αp50
αp65
17β-E2
- + - + - + - + - + - + - + - + - + - +
S2
S1
A
B
C
lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 5b.


Antibody
none
αp50
αp65
αER
Extract/ protein
+/+LDA11
p50
ER
TNF/IL-1
17β-E2
S2
S1
A
B
C
lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 95/06524

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 07072 (LA JOLLA CANCER RESEARCH FOUNDATION.THE REGENTS OF THE U. CALIFORNIA) 30 April 1992 cited in the application ---	
A	THE EMBO JOURNAL, vol. 9, no. 6, 1990 pages 1897-1906, S. AKIRA ET AL. 'A nuclear factor for IL-6 expression is a member of a C/EBP family.' cited in the application --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 September 1995	10. 10. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cartagena y Abella,P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. IMMUNOL., vol. 142, no. 9, 1 May 1989 pages 3134-3139, S. S. TABIBZADEH ET AL. 'Cytokine-induced production of IFN-B2/IL-6 by freshly explanted human endometrial stromal cells.' cited in the application ---	
P,A	MOLECULAR ENDOCRINOLOGY, vol. 9, no. 4, April 1995 pages 401-412, E. CALDENHOVEN ET AL. 'Negative cross-talk between RelA and the Glucocorticoid receptor: a possible mechanism for the antiinflamatory action of glucocorticoids. ' -----	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 95/06524

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9207072	30-04-92	AU-A-	8946991	20-05-92
		CA-A-	2093811	11-04-92
		EP-A-	0552302	28-07-93
		JP-T-	6502532	24-03-94